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*THE METABOLISM OF ISOPRENOID PRECURSORS IN A PLANT SYSTEM**

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Communicated September 21, 1954

Introduction.—It has been shown earlier[1, 2] that the synthesis of the isoprenoid, rubber, by a rubber-bearing plant, guayule (*Parthenium argentatum*), involves acetate as an intermediate. Not only does the administration of acetate to this plant result in a net increase in the amount of rubber formed, but, in addition, rubber synthesized by plants supplied with doubly C^{14}-labeled acetate possesses the same specific activity as the acetate given, indicating that all the carbon atoms of the rubber derive from and channel through acetate.[3] Rubber, like other isoprenoids, is made up of repeating units of isoprene, which has the five-carbon branched-chain structure shown below. This structure appears, then, to be derived in the plant from acetate. It has also been shown in earlier work[1, 2] that the five-carbon acid β-methyl crotonate (BMC) is able to replace acetate in supporting rubber formation and is, in fact, more effective than acetate in this function. BMC, which is known as a naturally occurring plant constituent,[4] has been shown to become radioactive in plant tissues supplied with C^{14}-labeled acetate,[3] and it has been suggested that BMC arises from acetate. This paper is concerned with the pathway by which BMC is synthesized from acetate by a plant enzyme system. The data to be presented suggest that the reactions involved follow the general course outlined below.

Route of Synthesis of β-Methyl Crotonate in a Plant System

$$\underset{\text{Acetate}}{CH_3COOH} + CoA \leftrightharpoons \underset{\text{Acetyl-CoA}}{CH_3CO\text{-}CoA} \tag{1}$$

$$2\ \underset{\text{Acetyl-CoA}}{CH_3CO\text{-}CoA} \leftrightharpoons \underset{\text{Acetoacetyl-CoA}}{CH_3COCH_2CO\text{-}CoAO} + CoA \tag{2}$$

$$CH_3COCH_2CO\text{-}CoA + H_2O \leftrightharpoons CH_3COCH_2COOH + CoA \tag{3}$$

$$\underset{\text{Acetoacetate}}{CH_3COCH_2COOH} + \underset{\text{Acetyl-CoA}}{CH_3CO\text{-}CoA} \leftrightharpoons \underset{\beta\text{-OH, }\beta\text{-Methyl glutaryl-CoA}}{\begin{matrix} CH_3 \\ HO \end{matrix}\!\!>\!C\!<\!\begin{matrix} CH_2COOH \\ CH_2CO\text{-}CoA \end{matrix}} \tag{4}$$

$$\underset{\beta\text{-OH, }\beta\text{-Methyl glutaryl-CoA}}{\begin{matrix} CH_3 \\ HO \end{matrix}\!\!>\!C\!<\!\begin{matrix} CH_2COOH \\ CH_2CO\text{-}CoA \end{matrix}} \leftrightharpoons \underset{\beta\text{-Methyl crotonyl-CoA}}{\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!C = CHCO\text{-}CoA} + CO_2 + H_2O \tag{5}$$

The key intermediate in this series of reactions is the substance β-hydroxy-β-methyl glutarate (BOG). This substance was first suspected as an intermediate in BMC synthesis when it was found by Bandurski *et al.*[5] to be a product of the metabolism of acetate by a leaf enzyme preparation. Bloch has suggested BOG as a possible intermediate in the synthesis of the isovaleryl carbon skeleton from acetate,[6] and Rabinowitz and Gurin have demonstrated the ability of a liver preparation to synthesize BOG from acetate.[7]

Materials and Methods.—Flax seedlings were the source of the enzyme system used in this work. It has been shown that flax seeds contain considerable amounts of BOG,[8] and flax was found to be more active in synthesizing BOG than other plants investigated in the present work. The plant material was grown from seed in vermiculite supplied with Hoagland's nutrient solution. The material was harvested after three weeks of growth under conditions of a 16-hour day (26° C.) and an 8-hour night (20° C.).

For the preparation of the enzyme, 40 gm. (fresh) of the apical inch were ground for 5 minutes in a mortar containing 5 gm. of washed sea sand and 80 ml. of a grinding medium modified from Rabinowitz and Gurin[9] and made up as follows: K_2HPO_4, 0.067 *M*; KH_2PO_4, 0.042 *M*; $MgCl_2$, 0.006 *M*; nicotinamide, 0.03 *M*; sucrose, 0.30 *M*. The resultant brei was strained through cheesecloth and the filtrate centrifuged at 500 × g for 10 minutes to remove debris. The greenish-yellow supernatant was then centrifuged at 30,000 × g for 40 minutes. The resultant pellet was triturated with four volumes of water and allowed to stand for 1 hour, after which the suspension was recombined with 40 ml. of the supernatant. The activity of the system appears to depend upon the presence of enzymes from the lysed particulate matter as well as upon components of the soluble fraction. The preparation of the enzyme was carried out at 1° C.

Reaction mixtures were incubated in stoppered 250-ml. Erlenmeyer flasks, in general, for 16 hours at 23° C. The standard reaction mixture included, in addition to substrates, ATP, GSH, and 5 ml. of enzyme (containing 4–5 mg. of protein per milliliter) in a total volume of 7 ml. BOG, BMC, and acetoacetate were added in the amount of 0.1 *M* to the reaction mixture. Radioactive substrates (1-C^{14}-acetate, 3-C^{14}-BMC, and $KHC^{14}O_3$) were used in the amount of 5 μM per reaction vessel. At the end of the experimental period, 2 ml. of reaction mixture were removed and added to an equal volume of saturated 2,4-dinitrophenylhydrazine in 2 *N* HCl for removal of carbonyl-containing compounds. To the remainder of the reaction mixture, 2 ml. of 1 *N* KOH were added and the solution allowed to remain for 1 hour at room temperature for hydrolysis of acyl-CoA derivatives. The solution was then adjusted to pH 2 and centrifuged, and the supernatant was extracted continuously with ether for 24 hours for removal of organic acids.

BOG was isolated from the ether extract of the reaction mixture by use of a silicic acid column prepared according to Bulen *et al.*,[10] with 10 per cent butanol in benzene as the eluant. The organic acids could also be separated by two-dimensional ascending paper chromatography, using 95 per cent ethanol, ammonia, and water (80:5:15), and tertiary amyl alcohol, formic acid, and water (65:30:5) as the solvents. Tentative identification of BOG was based upon coincidence of radioactivity in the column eluate or on the paper with authentic carrier material. The radioactive material eluted from chromatograms was also mixed with authen-

tic BOG and crystallized from a mixture of ether and ligroin. Repeated recrystallization gave no significant change in specific activity. BMC was isolated on one-dimensional chromatograms, using the alkaline solvent. To confirm the identity of the material, the eluted substance together with carrier BMC was dried *in vacuo*, sublimed, and then rechromatographed. The activity was confined to the BMC area of the chromatogram.

The 2,4-dinitrophenylhydrazones were extracted from the reaction mixture with chloroform and separated on paper using as a solvent 1.5 *M* $(NH_4)_2CO_3$ and 1.5 *M* NH_4OH (1:1).

Experimental Results.—The flax system is capable of converting the carbon of C^{14}-labeled acetate into citrate, acetoacetate, BOG, and BMC, as shown in Table 1.

TABLE 1

INCORPORATION OF 1-C^{14}-ACETATE INTO BOG, BMC, AND RELATED MATERIALS BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS*

	Counts/Min per Reaction Mixture			
System	BOG	BMC	Citrate	Acetoacetate
Complete	1,000	3,500	9,000	8,400
No ATP	400	2,000	900	4,100
No CoA	800	2,500	10,300	3,600

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM acetate containing 2.5×10^5 counts/min; and with or without 60 μM each of ATP and CoA, as indicated. Total volume, 7.0 cc.

These conversions are dependent upon the presence of added ATP in the reaction mixture and are, in general, increased in amount by the presence of added CoA. The unpurified system must be expected to contain both these substances to some degree. The synthesis of both citrate and acetoacetate in plant and other systems has already been extensively investigated and is relatively well understood.[11, 12, 13] Both reactions depend upon the formation of acetyl-CoA from acetate. The present system possesses the ability to activate acetate in the presence of CoA and ATP, as is shown in Table 2. For this experiment the reaction mixture was in-

TABLE 2

ACTIVATION OF ACETATE, BOG, AND BMC BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS*

	μM Activated per Reaction Mixture per 3 Hours		
System	Acetate	BOG	BMC
Complete	0.32	0.77	0.77
No ATP	.50	.37	.31
No CoA	0.07	0.50	0.07

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM substrate; and with or without 60 μM each of ATP and CoA, as indicated. Total volume, 7.0 cc.

cubated with substrate for 3 hours, and hydroxylamine was then added to convert the acetyl-CoA formed to acetyl hydroxamate, which was then determined colorimetrically.[14] The flax enzyme system contains an endogenous source of oxalacetate (malate) formed by a carboxylation reaction in the presence of ATP, and citrate is synthesized by union of acetyl-CoA with oxalacetate in the presence of condensing enzyme.[11] The synthesis of acetoacetate by the flax system indicates the presence of an acetoacetate cleavage enzyme capable of synthesizing acetoacetyl-CoA from acetyl-CoA.[13] The acetoacetate recovered in the reaction mixture accumu-

lates, however, as the free material rather than as the CoA derivative, indicating the presence in the reaction mixture of an acetoacetyl-CoA deacylase.

The BOG and BMC formed by the flax system do not accumulate as the respective free acids or salts but rather as derivatives which may be converted to the free acids by alkaline hydrolysis under conditions suitable for the hydrolysis of acyl-CoA derivatives (0.28 *N* KOH for 1 hour). The evidence suggests strongly that it is the CoA derivatives of BOG and BMC which are involved in the present reaction. The same conclusion has been arrived at on other grounds for the metabolism of BMC by liver (Robinson *et al.*[15]) and for the metabolism of BOG by a heart preparation by Bachhawat *et al.*[16]

The data of Table 1 show that with this system the accumulation of carbon from acetate in BMC is greater than in BOG. The accumulation of activity in BMC exceeded that in BOG by two to five times in separate experiments under varying experimental conditions.

That acetoacetate participates as an intermediate in the synthesis of BOG and BMC is indicated by the data of Table 3. In this experiment the enzyme

TABLE 3

PARTICIPATION OF ACETOACETATE IN BOG AND BMC SYNTHESIS AS INDICATED BY DILUTION OF 1-C^{14}-ACETATE INCORPORATION INTO THESE MATERIALS BY ACETOACETATE*

	COUNTS/MIN PER REACTION MIXTURE		
SYSTEM	BOG	BMC	Citrate
1-C^{14}-acetate	1,000	3,500	9,000
1-C^{14}-acetate + acetoacetate	200	1,900	7,000

* Reaction mixture contains 5 ml. enzyme; 40 μM DNP; 60 μM ATP; 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM acetate containing 2.5×10^5 counts/min; and with or without 50 μM acetoacetate. Total volume, 7.0 cc.

system was supplied with acetoacetate as well as with C^{14}-labeled acetate. The appearance of C^{14} in BOG and in BMC was depressed by the presence of the acetoacetate. This is not due to any considerable conversion of acetoacetate (through acetoacetyl-CoA) to acetyl-CoA, since the conversion of acetate carbon to citrate is depressed but little by the presence of acetoacetate. Incorporation of acetate carbon into BOG, on the contrary, was depressed two- to fivefold by acetoacetate and that in the BMC by about 50 per cent. The results are in qualitative agreement with those to be expected on the basis of reaction (4) above.

The sequence of reactions (4) and (5) above can be approached in the reverse direction, that is, from the BMC side. Thus, as shown in Table 4, the flax system synthesizes BOG from BMC and C^{14}-labeled CO_2. This synthesis is dependent upon the presence of added ATP and CoA (Table 4). The labeled BOG as well as the labeled BMC formed are present in the reaction mixture as alkali hydrolyzable derivatives, presumably the corresponding acyl-CoA compounds. It has been shown earlier that BMC is activated to form β-methyl crotonyl-CoA by plant systems,[13] and the data of Table 2 show that the present system activates both BOG and BMC in the presence of ATP and CoA.

When flax enzyme BMC and C^{14}-labeled CO_2 are incubated in a complete reaction mixture, labeled BMC appears as well as labeled BOG. This is not explicable in terms of reaction (5) alone. It may indicate that the free carboxyl group of

BOG becomes equilibrated with the acyl-CoA group, perhaps by a side reaction involving the cleavage and resynthesis of BOG-CoA. Alternatively, the carboxylation reaction may be more complex. That this is possible is indicated by the requirement of the carboxylation reaction for ATP reported by Bachhawat *et al.*[16]

The data of Table 5 show that the reverse of reaction (5), synthesis of BOG-CoA from BMC-CoA and CO_2, may be coupled to the reverse of reaction (4) to yield acetoacetate. This may be done either with unlabeled BMC and C^{14}-labeled CO_2 or with labeled BMC (3-C^{14}-BMC) and unlabeled CO_2. In both cases labeled acetoacetate appears in an amount considerably exceeding that of the BOG formed. In both types of experiment, the reactions formulated in (4) and (5) above would be expected to result in labeled acetoacetate, as was found, and in unlabeled acetyl-CoA. The data of Table 5 show, however, that a significant amount of labeled acetyl-CoA was recovered in the form of citrate. It is difficult to account for the presence of the labeled citrate in the reaction mixtures which contains 3-C^{14}-labeled

TABLE 4

SYNTHESIS OF BOG FROM BMC AND $C^{14}O_2$ BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS AS INFLUENCED BY ADDED ATP AND CoA*

SYSTEM	COUNTS/MIN PER REACTION MIXTURE BOG	BMC
Complete	1,400	2,000
No ATP	735	200
No CoA	130	67

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM ATP and 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM BMC; 5 μM $C^{14}O_2$ containing 3.0×10^5 counts/min; and with or without ATP and CoA, as indicated. Total volume, 7.0 cc.

TABLE 5

DEGRADATION OF BMC BY AN ENZYME SYSTEM FROM FLAX SEEDLINGS IN THE PRESENCE OF $C^{14}O_2$*

SYSTEM	COUNTS/MIN PER REACTION MIXTURE BOG	Acetoacetate	Citrate
BMC + $C^{14}O_2$	1,400	4,200	600
3-C^{14}-BMC + CO_2	1,610	3,100	460

* Reaction mixture contains 5 ml. enzyme; 40 μM DPN; 60 μM ATP; 60 μM CoA; 60 μM GSH; K phosphate buffer, pH 7.0, 0.05 *M*; 5 μM substrate; and 5 μM CO_2. Total volume, 7.0 cc. Total activity of $C^{14}O_2$, 3×10^5 counts/min. Total activity of 3-C^{14}-BMC, 2.5×10^5 counts/min.

BMC and CO_2 as substrates on grounds other than the activation and cleavage of a small portion of the acetoacetate to yield labeled acetyl-CoA.

Discussion.—It was suggested some years ago[2] that the synthesis of BMC might take place by the union of acetate with acetone. This was based upon the observation that both acetoacetate and acetone are effective in inducing net synthesis of rubber in intact rubber plants. These observations are, however, not incompatible with the pathway of BMC synthesis suggested by the present work, in which acetoacetate is also a metabolite. The role of acetone in the synthesis of isoprenoids has not yet been further elucidated in a plant system. It is possible that this substance may be recarboxylated to acetoacetate in the plant as it is in liver.[17]

The reactions involved in the synthesis of BOG and BMC by the flax enzyme system are very small-scale reactions as compared, for example, to those of the respiratory metabolism. The reaction mixture which synthesizes as much as 0.2 μM of citrate from acetyl-CoA and oxalacetate in a given time accumulates but one-tenth this amount of BOG in this same period. The same is true of other plant

systems, including guayule and lemon grass (*Cymbopogon citratus*), which have been found to synthesize labeled BOG from C^{14}-labeled acetate.

It has been shown above that the flax seedling, as well as a variety of other plant preparations, can activate and metabolize BMC. The flax system, unlike other plant systems investigated (spinach, guayule, bean, and lemon grass), is also able to activate BOG. It may well be, therefore, that free BOG as such is not an important metabolite in plant tissues in general but that it is rather the active BOG-CoA which is of significance.

The fact that the carbon atoms of steroids, such as cholesterol and ergosterol, are derived from labeled acetate[6] has led to studies of the manner in which acetate is converted to these isoprenoid-like compounds by animal tissues. In this case, too, BMC appears to be an intermediate and is synthesized from acetate.[18] In liver, also, BOG has been shown to arise from acetate.[19] For the liver as for plant systems the mechanism by which β-methyl crotonyl units are further converted to the higher isoprenoids has still to be worked out.

Summary.—

1. The mechanism of synthesis of branched-chain compounds from acetate has been investigated with the aid of an enzyme system prepared from flax seedlings.

2. It has been shown that this enzyme system is capable of activating acetate to acetyl-CoA and that the acetyl-CoA thus formed is converted to derivatives of β-hydroxy-β-methyl glutarate and β-methyl crotonate. The synthesis of β-hydroxy-β-methyl glutarate appears to take place through the acetylation of acetoacetate.

3. β-methyl crotonate is activated by the enzyme preparation in the presence of ATP and CoA and is converted with the uptake of CO_2 to β-hydroxy-β-methyl glutarate. The latter can be further degraded to acetoacetate and acetyl-CoA.

4. The sequence of reactions studied is of interest in relation to the role of β-methyl crotonate in the biosynthesis of rubber, steroids, and other isoprenoid compounds.

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*THE PRODUCTION OF MUTATIONS IN DROSOPHILA BY TERTIARY-BUTYL HYDROPEROXIDE**

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The mutagenic action of certain organic peroxides was first shown in work of Wyss, Stone, *et al.*[1] in 1947–1948 on *Micrococcus pyogenes* (*Staphylococcus aureus*) and, soon afterward, in work of Dickey, Cleland, and Lotz[2] in 1948 on *Neurospora crassa*. Because of the importance of peroxides in the production of mutations in microörganisms and as intermediates in the production of mutations by ultraviolet light, it seemed worth while to expand further the data concerning the mutagenic effectiveness of peroxides by exposing *Drosophila* to one of the more stable organic peroxides (*tertiary*-butyl hydroperoxide) and testing the offspring for the presence of mutations. The difficulty of exposing directly the reproductive cells of higher organisms to chemical mutagens has considerably delayed the study of mutagenesis. Of the higher forms, *Drosophila* is a particularly valuable organism because of its well-known genetic background and the important role it has hitherto played in the study of radiation mutagenesis. Chemical agents have in the past been applied to *Drosophila* by several methods: mixing the chemical with the food, spraying the flies, using the chemical as an aerosol, injecting or inserting the chemical into the hemocoele, applying it as a vaginal douche before copulation[3] or immediately after as a "sperm bath,"[4] and the laborious method of treating the entire gonad after its removal from the larva. Of these, the sperm-bath method is the most direct. Another reasonably practical method, accomplishing almost direct contact of the chemical agent with the germ cells, without operative procedure, involves treatment of early embryos after the fashion used by Edgar Altenburg[5] for ultraviolet light. The result of the experiments here described has been to demonstrate that, when *tert.*-butyl hydroperoxide is applied by this method, it produces a significant increase over the spontaneous lethal mutation rate in the second pair of autosomes in *Drosophila melanogaster*, although efforts to induce lethal mutations in the X chromosome by exposure of the adult flies to the peroxide failed.

Methods.—In the first series of experiments lightly etherized newly hatched adult *y v* males were placed in a glass jar lined with moist blotting paper and containing a dish filled with 5 ml. of a 50 molar per cent solution of *tert.*-butyl hydroperoxide in dibutyl phthalate covered with cotton gauze. The jar was glass-stoppered, and the flies were exposed to the peroxide vapor for varying lengths of time. The surviving males were mated individually to females of a balanced stock constructed

by H. J. Muller of composition *y* sc^{S_1} *B f In 49 v* sc^{8}/*y* ♀ *s*·Y^{S}. The Bar daughters were mated individually to normal males. The absence of non-Bar sons from a culture would indicate that a lethal mutation had been produced in an X chromosome of his treated father (crossing-over between the two X's being suppressed by the combination of inversions of the sc^{8} type and by the presence of *In 49* in the middle of the chromosome).

In the second series of experiments dechorionated fertilized eggs in the polar cap stage of development were either immersed in various dilutions of a 50 molar per cent solution of *tert.*-butyl hydroperoxide in dibutyl phthalate or were exposed to its vapor in closed containers. The control eggs were immersed in or exposed to the vapor of the same dilutions of dibutyl phthalate in mineral oil. In some instances the eggs were totally immersed in or exposed to the above agents, but more often all but the ends of the eggs containing the polar cap cells (the early germ cells) were shielded from the substances by placing the eggs in small holes in aluminum foil in such a manner that only the polar cap tips projected. In the immersion experiments a drop of the agent was placed on the projecting tips of the eggs for a period of 5 minutes and then washed off with mineral oil. In the vapor experiments, the foil containing the eggs was placed over a dish of water, with the body of the egg pointing downward, and sealed with Vaseline, in order to prevent drying out of the eggs and to protect them from the peroxide vapor. A dish containing 1 ml. of the agent being tested was placed a short distance above the foil. The whole apparatus was then sealed in a glass-stoppered jar for varying lengths of time.

After the eggs hatched, males carrying speck (*sp*) as a marker in one member of the second pair of autosomes and nonspeck (sp^{+}) in the homologous chromosome were selected and tested for the presence of lethal mutations by the *sifter* technique.[6]

Results.—In the first series of experiments an exposure of the adult males to peroxide vapor for 24 hours resulted in the death of nearly all the flies, but after 12 hours' exposure about half the males survived. From 28 males exposed in the adult stage for 12 hours a lethal mutation rate in the X chromosome of 0.2 per cent (1 lethal in 502 chromosomes) was obtained. No lethals were found in 133 chromosomes tested from 7 control males (that had been kept in a damp closed jar without peroxide). Although too few control males were tested in this experiment to be significant, the control mutation rate from a simultaneous experiment with the same stocks, involving treatment by ultraviolet light, was 0.163 per cent (2 lethals in 1,230 chromosomes, 68 males). Therefore it is apparent that peroxide treatment of the adults by this method is not mutagenically effective.

In the second series of experiments, *Drosophila* eggs in the polar cap stage of development were treated with peroxide. It will be seen from Table 1 that a lethal mutation rate of 5.7 ± 2.2 per cent (30 lethals in 531 tested chromosomes) was obtained from all the eggs receiving peroxide treatment, the control rate being 0.5 ± 0.5 per cent (1 lethal in 184 chromosomes). Thus the mutation rate induced by the the treatment was 5.2 ± 2.3 per cent. Although a small number of control males (those exposed to phthalate without peroxide) was tested, the control rate of 0.5 per cent (1 in 184) is within statistical range of the lower rate of about 0.2 per cent obtained from a great many controls tested in various experiments with ultraviolet light, and it is considered to be sufficiently accurate. Eight of the 21 treated males yielded lethals, and 4 of these males contained lethals in both the *sp* and sp^{+} chro-

mosomes, making a total of 12 independent lethals in 21 treated flies. The number of induced independent lethals per treated genome was 0.26. These figures correspond roughly to those obtained when *Drosophila* eggs are irradiated by us with a dose of 1,500 ergs/cm.^{2} of ultraviolet light of 2537 A wave length.

Because of the small number of eggs treated in these experiments, it is impossible to draw definite conclusions regarding dosage. However, it appeared that a 40-minute exposure either to undiluted peroxide vapor or to a 1:2 dilution of peroxide vapor is mutagenically the most effective dose, since a gross mutation rate of 8.4 per cent was obtained from 9 males and 286 treated chromosomes, whereas only

TABLE 1

LETHAL MUTATIONS PRODUCED BY *Tert.*-BUTYL HYDROPEROXIDE

Treated Series				Control Series			
		Number of F_3 Cultures (Mutants Shown in Parentheses)				Number of F_3 Cultures (Mutants Shown in Parentheses)	
Exp.	Treated Male No.	*sp*	*sp*$^+$	Exp.	Untreated Male No.	*sp*	*sp*$^+$
Immersion of Eggs in Peroxide				Immersion of Eggs in Phthalate			
3	2	28(1)	13(2)	1	1	12	9
5	1	14(14)*	21(1)	2	2	7(7)*	6
7	1	7	9	4	2	8	9
8	1	20(1)	20	6	1	10(10)*	15
Peroxide Vapor Treatment of Eggs				Phthalate Vapor Treatment of Eggs			
3V	1	18	8	2V	1	15	15
4V	1	12	7	4V	1	31	16(1)
	4	—	3	5V	2	15	13
	5	4	11		3	8	12
	6	8	5			—	—
	7	5	3	8 untreated males		89	95(1)
	8	14	8	Control mutation rate—0.5 ± 0.5 per cent			
	9	13(1)	8				
5V	1	9(1)	12				
	2	10(7)	11(4)				
6V	1	12	16				
	3	30(1)	36(9)				
	5	13	24				
	6	17	11				
	7	20(1)	21(1)				
	9	16	11				
	10	10	7				
21 treated males		266(13)	265(17)				

Treated mutation rate—5.7 ± 2.2 per cent
Mutation rate induced by treatment—5.2 ± 2.3 per cent

* Pre-existing lethals, not counted in the totals.

1 lethal was produced in 101 chromosomes (7 treated males), or a rate of 0.8 per cent, when the eggs were treated for 30 minutes with the vapor from a 1:2 dilution of peroxide. A rate of 4.2 per cent was obtained from eggs immersed in peroxide (5 lethals in 118 chromosomes), 4 treated males being tested.

Summary.—*Tert.*-butyl hydroperoxide has been shown to be capable of inducing lethal mutations in the second chromosome of *Drosophila* by treatment of the germ track at the polar cap stage of development of the egg, as measured by the sifter technique. The induced mutation rate obtained by the method of treatment used was 5.2 ± 2.3 per cent. By contrast, no mutagenic effect was detected when the males were treated in the adult stage.

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THE ANTIGENS DETERMINED BY THE H-2 LOCUS: A RHESUS-LIKE SYSTEM IN THE MOUSE

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On the basis of transplantation experiments, Snell and his coworkers have demonstrated that a series of alleles at the histocompatibility-2 locus (*H-2*) are decisive in determining the progressive growth or rejection of certain transplanted tumors in mice, more than seven different alleles having been discovered.[1, 2] In addition, the allele *H-2*^{d} of this series has been shown to be responsible for the presence of Gorer's isoantigen II in the cells of both normal and tumoral tissues,[3] and close linkage with the genes for tail defects Fused, Brachyury, and Kinky on chromosome IX has been demonstrated by these same authors. It has also been demonstrated that one of the alleles at this locus, *H-2*^{a} (formerly *H-2*^{dk}), behaves as a compound, possessing two components or factors which occur separately in *H-2*^{d} and *H-2*^{k}.[2] The purpose of this paper is to report an analysis of the antigenic differences in the alleles at the *H-2* locus based on red-cell agglutinins, using the technique developed by Gorer and Mikulska.[4]

The development by Snell[5] of isogenic resistant lines of mice (referred to hereafter as "IR" lines) has provided unique biological material for studying the antigenic differences which underlie the processes of resistance and susceptibility to tissue transplantation. As an IR line in theory differs from the line of origin only at one locus, that determining resistance to tumor transplants (and also, presumably, to normal tissues), the inoculation of tumors or normal tissues from a strain into its IR counterpart should give rise to an antibody directed only against that particular difference. This would make it possible to avoid the laborious techniques needed to separate in a serum the various antibodies that are formed in response to the inoculation of tissues into animals which differ in several antigens.[5] It is these IR lines and their strains of origin which have been used in the work reported here. Our findings have entirely confirmed the theory of Snell and his results of genetic tests of resistance to tumor transplants. However, the serological reactions are surprisingly complex, the situation in mice paralleling closely that described for the

Rhesus system in man[6] or similar systems in cattle[7] and chickens.[8] The *H-2* gene, or a system of closely linked genes, is responsible for the simultaneous presence in the cells of mice of two, three, and very possibly four different antigens which are detectable by red-cell agglutination.

MATERIALS AND METHODS

Strains of Mice.—In Table 1 the strains of mice and their IR counterparts which have been utilized are listed. Also included in the table are the *H-2* alleles of these mice as determined by Snell and his group, using transplantation techniques and tumors indigenous to the various strains that have been used in producing the antisera.

TABLE 1

MICE AND TUMORS USED IN THE PRODUCTION OF ANTISERA

Strain of Mice	*H-2* Allele	Indigenous Tumor	IR Line of Mice	*H-2* Allele	Indigenous Tumor
A/Sn	a	15091a	A.SW*	s	S786
AKR	k†	S775	AKR.K*	a	...
C57BL/10	b	B12a	C57BL/10-*H-2*^{d}‡	d	B11
C57BL/6	b	C1498	C57BL/6-*H-2*^{d}‡	d	...
DBA/1	q	S790	DBA/1B†	d	B8

* Unpublished data of G. D. Snell.

† Another mutation at the *H-2* locus in DBA/1 strain maintained by Borges and established as an IR line by selection. The designation used here is to be considered temporary, pending additional testing. Serologically *H-2*^{d}. (Unpublished.)

‡ Arose by spontaneous mutation at the *H-2* locus and established as IR lines by selection (G. D. Snell, P. Smith, and F. Gabrielson, *J. Nat. Cancer Inst.*, **14**, 481–484, 1953).

To determine the genetic nature of the antigens governed by the *H-2* locus, several reciprocal backcrosses have been made and the offspring tested for the presence or absence of certain antigens by means of appropriate sera. These crosses will be discussed below.

The Production and Collection of Antisera. All the antisera discussed here were obtained by the inoculation of normal or tumor tissue indigenous to a strain into its IR counterpart and vice versa. The results were identical whether normal or tumor tissue was used, in spite of the fact that many of the tumors employed have been transplanted through many successive transfers.

The normal tissues consisted of a mixture of lymphoid organs (axillary and inguinal lymph nodes, thymus, and spleen) and the liver from one animal each time. These organs were pooled and minced to the consistency of a fine mash. Without the addition of any diluent, 0.1 cc. of this mixture was drawn into a tuberculin syringe and inoculated intraperitoneally with a 19-gauge needle. This inoculation was repeated at 5–10-day intervals three or four times, depending on the titer of the serum observed after the third inoculation or the necessity for additional serum.

Tumors used are listed in Table 1. With the exception of tumors B8 and B11, which were inoculated subcutaneously by trocar, and B12, an ascites tumor, which was inoculated intraperitoneally, the first two inoculations of all tumors were made subcutaneously and subsequent ones intraperitoneally, using the sieve technique of Snell.[9] Four or more inoculations of tumor were given. The interval between inoculations was dependent upon the growth rate of individual tumors. Dosage was determined by cell counts and was increased with each inoculation until a dose

of several million cells was used for the third and fourth inoculations. The number of cells injected varied with the tumor, as previous experience had shown that strains of mice react differently to different tumors.

Five to seven days after the third and all subsequent inoculations, the mice were bled from the tail for the collection of antiserum. The yield of blood varied with age, the number of bleedings, and the strain, but it was usual by this procedure to collect 0.6–0.8 cc. of blood from each animal, and sometimes as much as 1 cc. could be taken at one time without causing the death of the animal. Blood was received into a clean tube, allowed to clot at 37° C., then centrifuged at 800 × g for 15 minutes. Blood for each antiserum and of each bleeding was pooled. A sample of the fresh antiserum was then tested, and, if the titer was sufficiently strong, the remainder was stored.

Although many antisera retain their strength for long periods of time when stored at −15° C., we have found that some, especially the weaker ones, may lose their specificity within a very short time. To avoid this, we now routinely lyophilize 0.1–0.2 cc. of serum in small tubes which are subsequently vacuum-sealed. In this condition, antisera will remain active for long periods of time, some having maintained the same titer for several months after storage at room temperature. To prepare lyophilized sera for use, the dried material is restored to the original volume by the addition of double-distilled water, and thereafter the procedure is the same as used for fresh antiserum.

Absorption.—The tissues used for absorption of antisera were a mixture of minced or homogenized liver, spleen, and thymus, washed three times with sterile 0.85 per cent saline and thoroughly packed by centrifugation at 800 × g for 10 minutes. Usually one absorption with one volume of tissue to one of serum diluted to $^1/_4$ or $^1/_8$ with Dextran was sufficient, but with some stronger sera two absorptions were necessary. The mixture of serum and tissue was placed in a 37° C. incubator for 1 hour, centrifuged, and the absorbed antiserum removed.

In specific cases, the antibodies were eluted from the absorbing tissues. For this, the tissues which had been used for absorption were washed three times in saline. Then a volume of 2 per cent Dextran in saline, equivalent to the volume of serum initially absorbed by the tissues, was added and allowed to stand at 56° C. for 15 minutes. The mixture was centrifuged for 1 minute at 1000 × g and the supernatant containing the eluted antibody removed. This supernatant was then used in the same manner as fresh serum. A constant zone of inhibition was observed with this procedure, which went up to $^1/_{16}$ to $^1/_{32}$ and prevented our using this technique with weak antibodies, i.e., anti-C.

The Agglutinating System.—The system used was that devised by Gorer and his coworkers,[4] which makes use of Dextran and human serum to enhance and show the agglutinations. It is important to mention here that not all Dextrans work well in this system, the results depending on the molecular weight of the Dextran and the source of production. Only one out of seven Dextrans tested by us proved satisfactory. This was a Dextran with a molecular weight of approximately 100,000.[10] Dextrans from other manufacturers, which had molecular weights of from 80,000 to 90,000, caused agglutination in the controls, while the one with the lowest molecular weight, 60,000, failed to show the presence of some antibodies, although it worked well with others.

It has been noted[4] that human sera may differ in their efficiency in this system. We have found that sera from laboratory workers who have long been in contact with mice have been unsatisfactory because high titers of antimouse antibodies are found in such sera and large quantities of mouse red cells must be used to absorb them. The best sera to use are those of healthy people who have never been in prolonged contact with mice. Using these, one absorption with an equal volume of washed mouse red cells at 37° C. for 1 hour is usually sufficient to obtain a satisfactory reagent.

For agglutination tests the mouse red cells were washed twice in 0.85 per cent saline and suspended in human serum/saline mixture (1:1) at a concentration of 2 per cent. Antiserum dilutions were made in Dextran diluted to 2 per cent with saline. Controls contained only Dextran, human serum, and mouse red-cell suspensions. The agglutination tubes contained Dextran/antiserum dilutions, human serum, and mouse red cells. The volumes used were the same as those used by Gorer, each agglutination tube containing 0.025 cc. of the antiserum in Dextran and 0.025 cc. of the red-cell suspension. The controls contained the same volumes of Dextran and red-cell suspension without the antiserum.

TERMINOLOGY

Because of the techniques involved in the identification of histocompatibility genes and the importance of these genes in transplantation, the problem of naming the antigenic systems which underlie these phenomena in the mouse presents special difficulties. No universal system of nomenclature has been established for isoantigenic systems and the genes responsible, in spite of the tremendous amount of literature on the subject. We are following, with modifications, the nomenclature suggested by Stormont, Owen, and Irwin for bovine blood groups.[7] This system is also closely related to that adopted by Briles for chicken blood groups.[8] The term "histocompatibility" replaces "blood" in their terminology.

1. *"Histocompatibility Factor" or Its Synonym, "Antigenic Factor."*—This designates the individual antigenic components, for which capital letters will be used, e.g., "D," "E," "K," etc. Although the present paper deals with only erythrocyte agglutinogens, it is known that some isoantigens may be present in tissues and leucocytes and not in the red cells.[11] We believe that the same terminology should be used in designating these. The reagents which define these antigenic factors will be called "anti-D" or "anti-K," etc., if the serum defines only one factor at a time, and "anti-DEK" or "anti-CES," etc., if the antiserum is multivalent.

2. *"Histocompatibility Groups."*—These are inherited characteristics within which several factors may be inherited as a unit. Thus the antigenic factors associated with *H-2*^{a} are D, E, and K, while those associated with *H-2*^{k} are C, E, and K. Synonyms for the term "histocompatibility group" are "antigens" and "antigenic group."

From the text it will be clear that we have no evidence to indicate whether we are dealing with the products of a single gene or of closely linked genes. Therefore, the antigenic factors making up any group are designated by capital letters in alphabetical order.

3. *"Histocompatibility System."*—Such a system contains groups which appear to be controlled by allelic genes; i.e., the entire constellation of *H-2* alleles would

make up one histocompatibility system. Here we have had to depart from the terminology of Stormont *et al.* In their nomenclature, alleles are designated by using characteristic blood factors as superscripts. In mice the study of tissue compatibility using tumor transplantation led to the discovery of strain differences in isoantigens prior to their identification by serological methods. It is probable that this transplantation technique will be used in the future for discovering such differences, and therefore we propose to retain the nomenclature for alleles already established by Snell. On occasion, it may be desirable to name alleles by their histocompatibility factors; e.g., *H-2*^{s} would become *H-2*^{CES}, in which case the gene symbols would indicate the antigenic makeup.

4. *"Histocompatibility Type."*—The histocompatibility type of any individual is the sum of its known histocompatibility groups—in other words, its antigenic phenotype.

RESULTS

The reactions of the different antisera with red cells from strains with known *H-2* genes are summarized in Table 2, and an account of the reactions of the antisera obtained in mutant IR lines is given in Table 3. The segregations of these antigens in crosses are shown in Tables 4, 5, and 6, and Table 7 gives a summary of the antigens of different *H-2* strains.

TABLE 2

SUMMARY OF RESULTS OF TESTING RED BLOOD CELLS OF MICE OF STRAINS WITH DIFFERENT *H-2* GENES WITH ANTISERA OBTAINED BY INOCULATING NORMAL OF TUMORAL TISSUES FROM INBRED STRAINS INTO THEIR ISOGENIC RESISTANT COUNTERPART AND VICE VERSA

		Reaction of Red Blood Cells with Antibodies against Antigenic Factors*								
Gene	Strains	B	C	D	E	K	P	Q	S	Antigenic Group
H-2^a	A/Sn; AKR.K	−	?	++++	+++	+++	−	−	−	C? DEK†
H-2^b	C57BL/10Bs; LP/Jax; C-57BL/6Sn	+++	−	−	+++	−	−	−	−	BE
H-2^d	C57BL/10-*H-2*^{d}; DBA/1B;‡ C57BL/6Ks; DBA/2; C57BL/6-*H-2*^{d}	−	++	+++	− −	−	−	−	−	CD
H-2^k	C57BR/a; AKR; C3H	−	++	−	+++	+++	−	−	−	CEK
H-2^p	P/Jax; P/Sn	−	++	−	+++	−	++	−	−	CEP
H-2^q	DBA/1	−	+±	−	++	−	−	++	−	CEQ
H-2^s	A.SW	−	++	−	+++	−	−	−	++++	CES

* ++++, +++, ++, +, stand for varying degrees of strength of agglutination; − indicates a negative reaction.

† We have not enough evidence of the existence of C in *H-2*^{a} strains.

‡ G. E. Foerster and P. R. F. Borges, *Transplantation Bull.*, **1**, 200–201, 1954.

1. *Serum A.SW anti 15091a.*—As was expected from the transplantation experiments, this serum reacted with *H-2*^{k}, *H-2*^{d}, and *H-2*^{a} red cells. Two antibodies, anti-D and anti-K, can be separated by absorption with tissues from the corresponding *H-2*^{d} or *H-2*^{k} strains, thus providing a reagent for each of these antigens alone.

2. *Serum A/Sn anti S786.*—This serum, unabsorbed, besides reacting strongly with A.SW red cells, cross-reacted less strongly with *H-2*^{b}, *H-2*^{d}, *H-2*^{k}, *H-2*^{p}, and

TABLE 3

REACTIONS OF RED BLOOD CELLS OF MICE WITH ANTISERA PRODUCED BY INOCULATION OF NORMAL OR TUMORAL TISSUES FROM TWO DIFFERENT MUTANT IR LINES AND THEIR STRAINS OF ORIGIN

	Absorbed by	Tested with Red Blood Cells from Mice of *H-2* Groups BE	CD	DEK	CEK	CEP	CEQ	CES
I. C57BL/10 anti C57BL/10-*H-2*^{d}	.....	−	+++	++++	+++	+++	++	+++
	C57BL/10	−	+++	+++	++	++	++	++
	C3H	−	+++	+++	−	−	−	−
	C57BL/10-*H-2*^{d}	−	−	−	−	−	−	−
II. C57BL/10-*H-2*^{d} anti C57BL/10	.....	+++	−	+++	+++	+++	++	+++
	C57BL/10-*H-2*^{d}	+++	−	+++	+++	+++	++	+++
	C3H	+++	−	−	−	−	−	−
	C57BL/10	−	−	−	−	−	−	−
III. DBA/1B anti S790*	.....	++	−	+++	+++	+++	++	+++
	DBA/1B	++	−	+++	+++	+++	++	+++
	A/Sn	−	−	−	−	−	++	−
	DBA/1	−	−	−	−	−	−	−

* DBA/1 tumor.

TABLE 4

SEGREGATION AND LINKAGE RELATIONS BETWEEN ANTIGENS D AND K*

Sex	Red Cells from	Mouse No.	A.SW anti 15091a Unabsorbed	Absorbed C57BL/10-*H-2*^{d} (Anti-K)	Absorbed C3H (Anti-D)
	A.SW		−	−	−
	A/Sn		+++	+++	+++
	C57BL/10-*H-2*^{d}		+++	−	+++
	C3H		+++	+++	−
♂	(A/Sn × A.SW) × A.SW	30	−	−	−
♂	(A/Sn × A.SW) × A.SW	32	−	−	−
♀	(A/Sn × A.SW) × A.SW	33	+++	+++	+++
♀	(A/Sn × A.SW) × A.SW	34	+++	+++	+++
♀	(A/Sn × A.SW) × A.SW	35	−	−	−
♀	(A/Sn × A.SW) × A.SW	36	+++	+++	+++
♀	(A/Sn × A.SW) × A.SW	65	−	−	−
♀	(A/Sn × A.SW) × A.SW	66	+++	+++	+++
♀	(A/Sn × A.SW) × A.SW	67	−	−	−

* Cross: (A/Sn × A.SW) × A.SW. (*H-2*^{a} × *H-2*^{s}). Antibodies: A.SW anti 15091a (anti-DK) absorbed with C57BL/10-*H-2*^{d} and with C3H (*H-2*^{k}). Reactions scored as in Table 2.

TABLE 5

SEGREGATION AND LINKAGE RELATIONS BETWEEN ANTIGENS K AND E*

Sex	Red Cells from	Mouse No.	A.SW anti 15091a Absorbed BALB/c (Anti-K)	C57BL/10-*H-2*^{d} anti-C57BL/10 (Anti-E)
	A.SW		−	+++
	C57BL/10-*H-2*^{d}		−	−
	C57BL/10		−	+++
	BALB/c		−	−
	C57BR/a		+++	+++
	(C57BR/a × BALB/c) × BALB/c			
♂	(C57BR/a × BALB/c) × BALB/c	55	+++	+++
♂	(C57BR/a × BALB/c) × BALB/c	56	−	−
♀	(C57BR/a × BALB/c) × BALB/c	57	+++	+++
♀	(C57BR/a × BALB/c) × BALB/c	58	−	−
♀	(C57BR/a × BALB/c) × BALB/c	59	+++	+++
♀	(C57BR/a × BALB/c) × BALB/c	60	+++	+++
♀	(C57BR/a × BALB/c) × BALB/c	61	−	−

* Cross (C57BR/a × BALB/c) × BALB/c. (*H-2*^{k} × *H-2*^{d} × *H-2*^{d}. Antibodies: A.SW anti 15091a absorbed with BALB/c tissues (anti-K) and C57BL/10-*H-2*^{d} anti C57BL/10, unabsorbed (anti-E) (this serum also contains anti-B antibodies, but this antigen is not present in the strains crossed). Reactions scored as in Table 2.

$H\text{-}2^q$ strains. The fact that this reactivity of the above-mentioned strains can be absorbed with A/Sn cells, leaving only the antibody which is reactive with A.SW cells, seems to indicate that this is a nonspecific cross-reaction. Therefore, we can assume that this serum defines the antigen S as determined by the $H\text{-}2^s$ allele.

3. *Serum C57BL/10 anti C57BL/10-H-2^d.*—This serum reacted with strains which carry the antigenic factor D ($H\text{-}2^d$ and $H\text{-}2^a$); it also reacted with several strains—$H\text{-}2^k$, $H\text{-}2^p$, $H\text{-}2^q$, and $H\text{-}2^s$—which do not contain the D factor. Agglutination with the red cells of these latter strains was definite but of medium or low

TABLE 6

SEGREGATION AND LINKAGE RELATIONS BETWEEN ANTIGENS K AND C*

Sex	Red Cells from	Mouse No.	C57BL/10 anti C57BL/10-$H\text{-}2^d$ (Anti-C)	A.SW anti 15091a (Anti-K)
	A.SW		+++	–
	C57BL/10-$H\text{-}2^d$		+++	+++
	C57BL/10		–	–
	AKR		+++	+++
♂	(AKR × C57BL/10)	1	+++	+++
♂	(AKR × C57BL/10)	2	+++	+++
♂	(AKR × C57BL/10)	9	+++	+++
	(AKR × C57BL/10)	3	–	–
♀	(AKR × C57BL/10)	4	+++	+++
♀	(AKR × C57BL/10)	5	+++	+++
♀	(AKR × C57BL/10)	6	–	–
♀	(AKR × C57BL/10)	7	–	–
♀	(AKR × C57BL/10)	8	+++	+++
♀	(AKR × C57BL/10)	10	+++	+++
♀	(AKR × C57BL/10)	11	+++	+++
♀	(AKR × C57BL/10)	12	–	–
♀	(AKR × C57BL/10)	13	–	–
♀	(AKR × C57BL/10)	14	+++	+++

* Cross: (AKR × C57BL/10-$H\text{-}2^b$) × C57BL/10-$H\text{-}2^b$. ($H\text{-}2^k$ × $H\text{-}2^b$) × $H\text{-}2^b$. Antibodies: C57BL/10 anti C57BL/10-$H\text{-}2^d$ (anti-C) (also contains anti-D, which is not present in the strains crossed) and A.SW anti 15091a (anti-K) (also contains anti-D). Reactions scored as in Table 2.

TABLE 7

SUMMARY OF *H-2* ALLELES, *H-2* ANTIGENIC FACTORS AND STRAINS CARRYING THEM AS SHOWN BY TUMOR TRANSPLANTATION AND RED BLOOD CELL AGGLUTINATION TECHNIQUES

H-2 Allele	Antigenic Factors	Strains
$H\text{-}2^a$	C* DEK	A/Sn; AKR.K
$H\text{-}2^b$	BE	C57BL/10; C57BL/6Bs and Sn; LP; C57L; 129
$H\text{-}2^d$	CD	C57BL/10-$H\text{-}2^d$; C57BL/6-$H\text{-}2^d$; C57BL/6Ks; DBA/ 1-B; DBA/2; B/10.D/2; BALB/c
$H\text{-}2^k$	CEK	C3H; AKR; C57BR/a; ST
$H\text{-}2^p$	CEP	P/Jax; P/Sn
$H\text{-}2^q$	CEQ	DBA/1
$H\text{-}2^r$	Not tested	RIII/WY
$H\text{-}2^s$	CES	A.SW

* Indicates C has not been completely proved to exist in this combination.

intensity compared with that observed with $H\text{-}2^d$ and $H\text{-}2^a$ strains The real existence of this new antigen, which we call "C" at the suggestion of Gorer, is proved by absorption experiments and by its dependence on the *H-2* locus as revealed by genetic data. After being absorbed with $H\text{-}2^k$ tissues, this serum reacted only with $H\text{-}2^d$ and $H\text{-}2^a$ strains. There was no nonspecific absorption with $H\text{-}2^b$ tissues, and everything was absorbed with $H\text{-}2^d$ tissues.

4. *Serum C57BL/10-H-2^d anti C57BL/10.*—This serum, the reciprocal of serum No. 3, gave us the unexpected finding of an antigenic factor not shown in trans-

plantation experiments. Besides reacting with all *H-2*^{b} strains, it agglutinated the red cells of all other strains tested except those of *H-2*^{d} strains. After being absorbed with *H-2*^{p}, *H-2*^{k}, or *H-2*^{a} tissues, only agglutinins for *H-2*^{b} strains were left. This antigen was discovered independently by Gorer with antibodies against his E. 4 line of leukemia, and thus it has been named "antigen E" (personal communication).

The same results as in Nos. 3 and 4 were obtained when the reciprocal antibodies were elicited with three different lines of tumors—B11 of *H-2*^{d} origin and B12a and C1498 of *H-2*^{b} origin—instead of using normal tissues from these two C57BL strains.

5. *Serum DBA/1B anti S790.*—Strain DBA/1B (*H-2*^{d}) arose probably by mutation at the *H-2* locus from strain *DBA*/1 (*H-2*^{q}) (Borges and Foerster, see note to Table 2). As can be seen in Table 3, this antiserum, besides reacting with DBA/1 red cells, cross-reacted with all strains carrying the E antigen. Absorption with *H-2*^{a} tissues removed all the cross-reactive antibodies and left a fluid which reacted only with DBA/1 cells; i.e., this serum defines antigen Q, characteristic of allele *H-2*^{q}. No nonspecific absorption was observed when DBA/1B tissues were used, and all antibodies were removed from the serum by absorption with DBA/1 tissues. This is further proof of the existence of antigen E in strain DBA/1. Thus we can conclude that in this case, as in the observed mutations at the *H-2* locus in the C57BL lines, two antigens have been affected at once. However, as DBA/1 red cells react with anti-C serum, the situation is not the same as in the C57BL lines, which have in their red cells either C or E antigens but not both simultaneously. The low titer of DBA/1 red cells with anti-C sera, as compared with the titer of other C carrying red cells with the same sera, indicates that the C factor in DBA/1 may be slightly different.

6. *Serum C3H anti P/Jax.*—Because strain P/Jax has no IR counterpart, sera for the study of the *H-2*^{p} allele had to be prepared in unrelated mice. For this purpose, C3H mice were selected because they were known to carry the C and E factors also present in P/Jax.

This antiserum was tested against red blood cells of strains A/Sn (*H-2*^{a}), BALB/c (*H-2*^{d}), C57BL/10 (*H-2*^{b}), C3H/Sn (*H-2*^{k}), DBA/1 (*H-2*^{q}), and P/Jax (*H-2*^{p}). It reacted strongly with P/Jax (1/512) and also with A/Sn (1/64) and was negative with the other four strains. Antibodies reacting with A/Sn were absorbed nonspecifically by C3H or C57BR/a tissues, the resulting fluid reacting only with P/Jax red blood cells. P/Jax tissues absorbed all antibodies present.

This is evidence that the *H-2* histocompatibility group of P/Jax mice is CEP. The serum, however, lost its potency before it could be tested against the whole panel of *H-2* cells, and conclusive proof of the existence of P must wait until a new serum is obtained.

7. *Serum AKR.K anti S775.*—The existence of other antigenic factors at the *H-2* locus not yet clearly identified by the methods used, but still important in tumor transplantation, is suggested by the reactions of IR mice AKR.K (*H-2*^{a}) against tumor S775 of strain AKR (*H-2*^{k}) origin. This tumor does not grow in AKR.K mice. The serum of the resistant animals agglutinates the red blood cells of *H-2*^{k} mice. The clumps, however, are extremely loose and friable, and the titer is low. A slight degree of cross-reaction with the red blood cells of strain BALB/c (*H-2*^{d}) was observed. As strains which carry *H-2*^{k} differ from *H-2*^{a} strains in not

having antigen D, we think that these reactions are caused by an antibody against an antigenic factor allelic to D; but, since the reactions with different batches of the antisera have given irregular results, we are not prepared to form any definite opinion until more consistent results are obtained.

To date, all strains tested belonging to the same *H-2* group have the same antigens, with slight variations in the strength of the reaction. As has been noticed by Gorer and Mikulska,[4] strain A/Sn and its IR counterpart, A.SW, respond with a higher titer to the same *H-2* antibodies than do other strains.

Data obtained by testing the antigenic specificities of the offspring from backcrosses of a given type with appropriate antisera (Tables 4, 5, and 6) show clearly that antigens D and K, K and C, and K and E are very closely linked. The antigenic nature of other strains, e.g., $H\text{-}2^s$ (CES), $H\text{-}2^d$ (CD), show that C and E can exist independently of K, precluding the possibility that they are the result of gene interaction, as seems to be the case for some isoantigens.[7] Whether this complex antigenic system associated with the *H-2* locus in mice is determined by a single locus or by closely linked genes will be discussed below.

DISCUSSION

The results presented above establish that the *H-2* system is responsible for the presence in the red blood cells and tissues of at least eight antigenic factors, of which three and probably four are proved to be genetically linked. In addition to the already known antigen D, our analysis has demonstrated the actual existence of most of the antigens postulated by Snell on the basis of transplantation experiments. Besides this, two and very probably three new antigenic factors which were not suspected were discovered. Different strains have different combinations of these antigens, although in some only two have been identified. However, all strains which have been classified as having identical *H-2* alleles on the basis of transplantation tests have been shown to have exactly the same antigens present (Table 7) These antigens have so far shown complete linkage. As we have already mentioned, the existence of B and of one of the three new antigens, E, have been independently discovered by Gorer and his coworkers, and their identity has been established by interchange of lyophilized sera (personal communication).

From the results in Tables 2 and 3 it may be seen that each of these antigens is a separate entity. When tested with the same antisera, most strains respond with a fairly uniform titer Exceptions to this situation are strains A/Sn and A.SW, which show a consistently stronger response for the *H-2* antigens they have in common with other strains, and DBA/1 mice, which have a definitely weaker and sometimes irregular response when tested for antigens C and E. However, the absorption of appropriate antisera with DBA/1 tissues shows antigens C and E to be present in this strain. Whether this is a quantitative phenomenon or whether strain DBA/1 has some antigens closely related to, but not identical with, C and E of the other strains, we cannot say at present. The consistently stronger response of A strain red cells has been noticed also by Gorer and Mikulska.[4] A/Sn tissues also seem to absorb more anti-E antibodies per volume than do tissues of C57BL/10. The titer of the agglutination in itself is only a comparative estimation of the antigenic content, as it can be altered by changing the concentration of the reagents used, especially the Dextran.

As far as present information goes, the factors B, Q, and S are absent when D or K or both D and K are present. On the contrary, C and E can be found in combination with any of the other factors or with each other. From the mutual exclusion of B, Q, and S as against D and K, it is suspected that these factors bear an allelic relation; but we have no way of deciding whether D or K or both are the basis of the series. A decision will be reached once a reliable antiserum is prepared in AKR.K mice (DEK) against the IR counterpart, AKR (CEK), therefore defining a non-D component. Should the allelic relation be established, a change in factor symbols might be indicated.

The association of many antigens with a unit which segregates as a single gene has been described in man[6] and in other species[7, 8] Thus the interpretation here presents the same problems encountered in the analysis of comparable systems: Are these antigenic complexes the product of a single gene, as Wiener postulates for Rh, the MNS, and other systems,[12] or are we dealing with closely linked genes with similar functions, as Fisher and Race[6, 13] contend? Aside from this important theoretical question, the relation known to exist between the *H-2* genes and tissue compatibility in transplantation poses other questions which are discussed below.

It can be seen in Table 3 that the reciprocal antisera between strain C57BL/10 and its mutant IR line C57BL/10-*H-2*^{d} show the existence of two different antibodies: anti-B plus anti-E and anti-D plus anti-C, which indicates that these lines *differ in two antigenic components.* The same situation holds true for the other two strains and the corresponding IR lines obtained by mutation: C57BL/6 and C57BL/6-*H-2*^{d} and DBA/1 versus DBA/1B (Borges and Foerster, see note to Table 2). The former was a mutation from *H-2*^{b} to *H-2*^{d}, as first observed in C57BL/10, and behaves serologically like the C57BL/10 couple. In the latter a mutation from *H-2r* to *H-2*^{d} took place. The agglutinations in this case show that the DBA/1B mutant IR line differs from the original strain, DBA/1, in two antigens, Q and E, which are lacking in the DBA/1B IR line.

According to Borges *et al.*[14, 15] these three IR lines have arisen through spontaneous mutation at the *H-2* locus, and these authors point to the possibility that this phenomenon "may occur more frequently than has been suspected."[15] Though the presumption that a mutation has been responsible for the changes in transplantability is well documented, we believe that on the basis of their own data it cannot be affirmed categorically to have happened. The appearance of rare resistant mice in an otherwise genetically homogeneous strain could also be accounted for by the residual heterozygosis of genes which have not been selected against. In the light of other information—the existence of lines such as C57BL/6Ks, which is *H-2*^{d}, and DBA/2, also *H-2*^{d}—and of the fact that Snell has reported that other sublines do not give satisfactory results with all *H-2* strain tumors, it would not seem impossible that some lines, in spite of many generations of inbreeding, still maintain some degree of heterozygosity as far as *H-2* factors are concerned. Occasionally mice belonging to strains inbred for more than thirty generations have been shown by MacDowell[16] and by Bittner[17] to resist a tumor to which all animals of these strains usually succumb. MacDowell remained in doubt about the nature of the resistance of the one animal which survived in over more than 3,000 inoculated with line A leukemia.

Bittner observed that 67 out of 341 mice of the highly inbred DBA/1 strain from Dr. W. S. Murray's stock failed to grow tumor dbrB indigenous to the DBA/1

strain of Dr. L. C. Strong's colony. As the tumor had not changed its histocompatibility requirements in relation to Strong's DBA/1 mice, he concluded that "a change has occurred within the genetic constitution of one or more of the mice, which by the continued inbreeding of the stock of dilute brown mice now in Dr. Murray's laboratory has given rise to a mutation among the factors which determine susceptibility to transplanted cancer." The bulk of all this evidence, plus the high number of alleles observed at this locus, seems to us to support the contention of Borges *et al.* that mutations of histocompatibility genes are not uncommon events.

If a mutation is really responsible for the observed changes of two antigens at once, this would strongly support Wiener's contention of the allelic nature of the inheritance of blood-group agglutinogens.[12] Because the probability of three double mutations occurring is so low that it can be excluded as an explanation, we feel that Fisher's concept of closely linked genes cannot be utilized to explain these three cases in which two factors have changed.

Additional evidence in favor of Wiener's contention is the fact that the changed antigens C and E are not alternative conditions. This conclusion is borne out by the fact that several inbred strains carry both C and E at the same time. If two mutations were responsible for the observed changes in antigenicity, one would expect them to behave in inheritance as alleles. This same observation would rule out crossing-over in double heterozygotes as an explanation for these changes. An additional argument in favor of the idea of one gene to account for the several antigens involved is the uniformity of the antigenic makeup of all the strains which carry the same *H-2* allele regardless of their origin: six $H\text{-}2^b$ and nine $H\text{-}2^d$ strains and all three $H\text{-}2^k$ strains tested so far carry the same set of antigenic factors each.

If crossing-over between *H-2* components is proved, we must resort to Fisher's concept of closely linked genes. However, should this be the case, we feel that it does not preclude the possibility of one gene determining multiple antigenic factors.

The association of the new antigens C and E with other antigens makes it difficult to ascertain their relative importance in tissue transplantation. Further study may show their role in histocompatibility. Dr. N. A. Mitchison has found in his work on tumor immunity a partial solution to this question (personal communication). When mice of a C57BL/10 IR line, B/10.D/2 (CD) immunized against C57BL/10 (BE), are challenged with a tumor of a third strain, C3H (CEK), the immune B/10.D/2 hosts reject the C3H tumor much more quickly than untreated B/10.D/2 mice, i.e., they exhibit a "second set" response instead of a primary response. Since the only *H-2* antigen in common between the immunizing tissue of C57BL/10 and the C3H tumor is E, the accelerated breakdown of the tumor graft is a measure of the efficiency of the E component to elicit a protective response. We can thus conclude that the E factor plays an active role in tumor transplantation. The reverse combination, C57BL/10 immunized against B/10.D/2 tissues and challenged with a C3H tumor, should show whether or not the C component would also elicit a protective response. For a more direct approach, however, we will have to wait for some unusual variant to have C or E only, as in the case described for the Rh system, in which only D was present.[18]

Some of the problems involved in the nomenclature of this system have already been mentioned and will be dealt with more extensively in a separate communication.

SUMMARY

A serological analysis of the histocompatibility-2 locus of the mouse has shown that, in addition to its role in tumor transplantation, this locus is responsible for a complex system of isoantigens. These isoantigens are found in the red blood cells, in normal and in neoplastic tissues, and are inherited *en bloc*.

In addition to the previously reported antigen D, seven more antigenic factors have been identified.

As previously demonstrated by tumor transplantation, some antigens are characteristic of certain strains of mice and others are shared in common by several strains.

Three mutations at this locus have each been shown to change two antigens simultaneously.

The relationship of these findings to tumor transplantation and their bearing on the interpretation of the genetic nature of similar systems existing in humans and in other species are discussed.

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*PRODUCTION OF MITOTIC ABNORMALITIES BY RIBONUCLEASE**

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The normal course of mitosis in plant and animal cells can be disrupted by many kinds of physical and chemical agents. Nuclear transformations essential to the orderly progress of mitosis can be retarded, chromosome structure deformed, spindle mechanisms impaired, and cytokinetic processes inhibited. Such disturbances may be revealed, on cytological examination of the treated tissues, by a low frequency of dividing cells or a preponderance of specific stages of mitosis; by the presence of fragmented, adherent, or excessively contracted chromosomes; or by the occurrence of aneuploid and polyploid chromosome complexes, tripolar and multipolar spindles, binucleate and multinucleate cells.[1]

Production of these types of aberrations by a variety of agents suggests that the same cellular constituents are ultimately affected, despite possible differences in the nature of the reaction system initiated by the inciting stimulus. In an effort to identify the structural materials involved, we have determined the capacity of some crystalline enzymes to induce mitotic abnormalities in growing roots of onion and lily. Preliminary experiments revealed that ribonuclease (0.01–0.1 per cent aqueous solutions) induced aberrations of divers types with a high frequency but that equimolar concentrations of trypsin, chymotrypsin, and deoxyribonuclease had little effect in modifying chromosome form and distribution.[2] Such differences in cellular response may be due, at least in part, to differences in ability of these four enzymes to penetrate the cell; ribonuclease has the lowest molecular weight (about 13,000, as compared with 20,000–60,000 for the others)[3] and might for that reason be expected to enter the living cell more readily.

An extensive analysis of the action of ribonuclease on meristematic root-tip cells was accordingly undertaken and forms the basis of the present report. This analysis has led to the conclusion that the production of mitotic abnormalities may be initiated by enzymatic degradation of the ribonucleic acid (RNA) component of ribonucleoproteins.

Experimental Methods.—Growing roots of onion (*Allium cepa* L.) and lily (*Lilium tigrinum* Ker.) were treated by immersion in aerated solutions of ribonuclease or other reagents that served as controls, for periods ranging from 15 minutes to 48 hours, at room temperature (*ca.* 22° C.). Seven different samples of ribonuclease were tested,[4] and all were effective in inducing mitotic abnormalities. Control solutions were selected to evaluate the action of the solvents and to determine whether the induced abnormalities could be attributed to properties of the ribonuclease solutions other than their enzymatic activity. Therefore, they included water, potassium phosphate buffer, ribonuclease that had been inactivated by boiling or exposure to ultraviolet radiation, a mixture of amino acids in the proportions characteristic of ribonuclease,[5] histone, protamine, egg albumin, sucrose, and a ribonuclease digest of RNA. Cytological diagnosis of the action of the enzyme and

control solutions was made by examination of squashes of the treated roots stained in aceto-orcein and of sections stained in basic and acidic dyes (used independently and in combination) or by the Feulgen method.

Results.—Treatment of roots in a solution of ribonuclease produced gross changes affecting the pattern of growth and of microscopically detectable changes affecting the structure and distribution of the chromosomes.

Alteration of the normal pattern of growth, as evidenced by looping and coiling of the roots, apparently reflected some modification in the distribution of growth-promoting substances. Further indications of such modification were afforded by the appearance of polyploid chromosome complexes in the region of cell elongation of some onion roots during the first few hours of treatment. Since the higher degrees of ploidy observed (8- and 16-ploid) could not have arisen within this short time interval from diploid cells through repeated doublings in successive mitoses, it is assumed that the action of the solution of ribonuclease stimulated division of cells that had attained a polyploid state in the course of differentiation.[6]

The effect of solutions of ribonuclease on cells of the meristematic region was revealed by the appearance of all the types of abnormalities noted in the introductory paragraph of this report. The nature and frequency of the induced alterations and the ability of the cells to resume mitotic activity were found to be related to the pH of the solution, the concentration of the enzyme, and the duration of the treatment. Details of the experiments on which these generalizations are based will be published elsewhere; a summary of the results is presented here.

The effect of pH was tested in the range between 5.6 and 7.9, using a 0.8 mg/ml solution of ribonuclease for 2–9 hours. At the extremes of this pH range aberrations were produced, but the solutions readily killed the cells. At pH's between 6.0 and 7.0, solutions of ribonuclease proved less toxic, although capable of inducing mitotic abnormalities. On the basis of the information gained in this survey, concentration and "recovery" studies were made at pH 6.0; ribonuclease dissolved in tap water gave essentially this pH, and any necessary adjustment was made by adding a trace of 0.001 N sodium hydroxide.

At pH 6.0, concentrations of ribonuclease of 0.001 mg/ml of solvent (8×10^{-8} M) had no apparent effect on mitotic processes even when treatment was continued over long periods of time. A tenfold increase in concentration did not inhibit mitosis but caused enlargement of the nucleoli and excessive contraction of the chromosomes after 24–48 hours' treatment. Abundant abnormalities were produced by 0.1 mg/ml solutions and by those of higher concentration.

Treatment for 2–4 hours with a 0.1 mg/ml solution caused excessive contraction and "stickiness" of metaphase and anaphase chromosomes. The latter effect was revealed by adhesion of the distal ends of sister-chromatids, which formed bridges between the separating anaphase and telophase groups of chromosomes. After 4–8 hours of treatment, cells were observed in which strongly contracted chromosomes with paired chromatids were scattered at random (the *c*-type mitosis characteristic of colchicine treatment) or aggregated into two or more groups whose members showed various degrees of stickiness. Prolonged treatment led to inhibition of mitosis and eventually to pycnotic degeneration of the cells.

The course of production of these abnormalities, which proceeds from the periphery across the root to the central axis, parallels the course of movement of the

enzyme, as revealed by a decrease in stainability of the cells with the basic dye pyronin and an increase in stainability with the acidic dye fast green. Thus a 0.1 mg/ml solution modified the colorability of cytosome and nucleus of cells in the outer layers of the root meristem within a few hours and eliminated pyronin stainability of all meristematic cells within 24–48 hours. The marked modifications of stainability effected by long periods of treatment reflect degradational changes of such magnitude that recovery of the cells is seldom possible; but, if the roots are transferred to water after a short exposure to the action of the enzyme, they may continue to grow, as indicated by the presence of dividing cells with various types of abnormalities, including polyploid chromosome complexes, chromatid bridges, multipolar spindles, and multinucleate cells. Chromosome fragments were rarely observed, and this finding suggests that solutions of ribonuclease do not effect direct breakage of chromosomes in cells of the root-tip meristem.

Solutions containing between 0.2 and 1.0 mg. of ribonuclease per milliliter of solvent also produced the types of aberrations noted above, but the time interval between their appearance and the termination of vital activities of the cell was shortened as the concentration of the enzyme was increased. It was thus possible to determine the immediate as well as the delayed responses of the cells at different stages of mitosis by comparing the effects of solutions of ribonuclease at different concentrations.

Among the controls, solutions of histone, protamine, and inactivated ribonuclease induced some types of aberrations, but with much lower frequencies than did equimolar solutions of ribonuclease. A few aberrations were also induced by ultraviolet-irradiated water. Other controls, including water, and solutions of egg albumin, sucrose, the mixture of amino acids in the proportions characteristic of ribonuclease, and the ribonuclease digest of RNA induced very few abnormalities in any of the treated roots.

Discussion.—The reaction pathways involved in the production of mitotic abnormalities by ribonuclease have not been determined. Ribonuclease might conceivably act as a basic protein to disrupt structural complexes or to interfere with synthetic processes.[7] The efficacy of histone, protamine, and even inactivated ribonuclease in producing some types of aberrations indicates that this possibility cannot be eliminated. On the other hand, evidence that ribonuclease may act as an enzyme within the living cell to degrade structural complexes containing RNA is afforded by the correlation between the appearance of aberrations and the modifications in stainability of the cells as the enzyme moves across the root. Stainability of RNA is reduced, but that of deoxyribonucleic acid (DNA) is not altered.

The degradation of structural nucleoproteins initiated by the action of ribonuclease represents one possible mechanism for the production of mitotic abnormalities. The need for intact nucleoproteins in maintenance of the structural framework of the cell was demonstrated in studies of the action of trypsin on fixed and unfixed salivary-gland cells of *Drosophila*.[8] Degradation leading to structural deformation might conceivably be initiated in the nucleic acid or protein moiety of either the ribonucleoprotein or the deoxyribonucleoprotein, but the results obtained in the experiments using ribonuclease focus attention on the RNA component.

Ribonucleoproteins are essential structural constituents of both cytosome and nucleus, and the ribonuclease-induced alterations affect chromosome distribution as

well as form. Many cytoplasmic particles are rich in ribonucleoprotein, and the spindles of onion root-tip cells contain both RNA and protein.[9] Appreciable amounts of RNA are present in the condensed chromosomes[10] and in the nucleolus.[11] Chemical analysis of wheat germ indicates that the concentration of nuclear RNA may be as high as that of DNA in actively dividing tissues.[12] It is thus apparent that on a purely quantitative basis ribonucleoproteins cannot be ignored in assessing the factors involved in the production of chromosomal aberrations and mitotic abnormalities. If, in addition, the native ribonucleoproteins are more labile than the deoxyribonucleoproteins, as seems probable from extraction and turnover studies of RNA and DNA,[13] they would provide a substrate responsive to the action of any agent capable of interfering with mitotic processes. Whether the degradation induced thereby would involve a direct action or an indirect pathway would appear to depend on the properties of the inciting agent.[14] In this connection, the following alternatives must be considered: that modifications in chromosome form and distribution may be initiated by degradation of ribonucleoproteins of cytoplasmic particles (for example, by ribonuclease or by acriflavine[15]) and the consequent functional impairment of their constituent enzymes, or, conversely, that structural modification may be initiated by impairment of enzymatic activity (for example, by enzyme poisons) and the disturbance of processes essential to the metabolism of RNA.

These considerations suggest that the production of mitotic abnormalities by agents other than ribonuclease may be attributable in part to degradation of ribonucleoproteins. A vast number of agents can inhibit spindle mechanisms and cause stickiness or clumping of chromosomes, whereas relatively few can induce chromosome breakage or promote structural rearrangement. The first group of responses characterizes the "primary" or "physiological" effects of ionizing radiations, whereas the second group typifies the "secondary" or "structural" effects. Ribonuclease readily produces responses of the first type, rarely those of the second. It has been suggested, accordingly, that the primary effects can be initiated by changes in RNA, whereas the secondary effects involve more profound alterations in nuclear materials, perhaps directly affecting deoxyribonucleoproteins.[16] In this connection, it has been reported recently that a given dose of X-rays is more effective in inducing changes that result in stickiness and clumping if given at late prophase than if given at metaphase or anaphase.[17] Maximum sensitivity thus seems to occur during the stages when RNA is accumulating in the chromosomes.[10]

Although the ribonucleoproteins appear to afford particularly vulnerable sites for the initiation of structural changes, the results obtained in treatment of roots with control solutions suggest that degradation of any component of the nucleoprotein complex may lead to production of aberrations. The changes effected by such degradation in the colloidal properties of cellular materials could conceivably underlie the concomitant modifications in chromosome form and distribution.

Summary.—Treatment of growing roots of onion and lily with solutions of crystalline ribonuclease stimulated mitotic activity in polyploid cells of the region of cell elongation and modified the form and distribution of chromosomes in the meristematic cells. The production of aberrations by ribonuclease is associated with the degradation of ribonucleoproteins, as is shown by the close correlation between the appearance of abnormalities and the modifications in stainability of the cells with

pyronin and fast green as the enzyme moves across the root. This correlation emphasizes the importance of ribonucleoproteins in determining normal chromosome form and distribution during the course of division of meristematic cells. The findings have been evaluated with relation to the action of other chemical and physical agents in producing similar types of mitotic abnormalities.

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DIFFUSION OF CHROMOGENIC INDUCTORS OF SERRATIA MARCESCENS

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Spontaneous and induced color variants in the red HY strain of *Serratia marcescens* have been reported by Bunting[1] and Labrum and Bunting.[2] Many of the color variants are stable, but unstable types have also been isolated. The expressivity of color in these variants as well as in the wild-type strain is influenced by environmental conditions, such as temperature and nutritional factors.[3] When three stable color variants, orange, pink, and white, were streaked side by side on a Petri dish merely to demonstrate the variety of mutations found in *Serratia*, it was noted that the margin of the white culture growing next to the streak of orange had turned red, and similarly the margin of orange facing the pink culture had become red. Apparently a diffusible factor produced by the orange variant had induced red pigmentation in white, and, similarly, a factor from the pink culture had caused the production of red pigment in the orange streak. Subsequent plating of the color-induced organisms showed that this induction is a phenotypic change and not permanent. In order to investigate the extent of this phenomenon in *Serratia marcescens*, a large number of colored mutants were tested for interaction type to determine whether a given variant was able to produce a factor which could induce color in other variants (i.e., was an *inductor*) or could react to an *inductor* by expressing a color change (i.e., was a *reactor*) or could be qualified both as an *inductor* and as a *reactor* or as neither.

All mutants used in the present investigation were induced by ultraviolet irradiation of the HY strain obtained from Dr. M. I. Bunting's laboratory. The mutants are divisible into three color types: white (*W*), pink (*P*), and orange (*O*). Within each of these classes there are differences in shade of color, surface texture, and general appearance of the culture on aging. Of the different variants, only those which can be characterized by the inductor-reactor system of red pigment are included in this report. These twelve mutants were chosen from a preliminary test of twenty-six variants by streaking different combinations of variants on Petri dishes in the form of a Punnett square.

Petri dishes were prepared with a peptone-glycerol agar medium, according to Bunting,[1] with 2.0 per cent agar. Similar results were also obtained with the synthetic ammonium-citrate-glycerol agar used by Bunting. A triangular piece of cardboard was used to draw two divergent lines on the bottom of each Petri dish with a wax marking pencil. The smallest distance between these guide lines measured approximately 2–3 mm., while the opposite sides were 40 mm. apart. Each variant of *Serratia* was suspended in a 0.9 per cent saline blank at approximately 10^8 concentration, and the suspensions were smeared on the Petri dishes using sterile cotton swabs. Each Petri dish was streaked with two variants, one on either side (Fig. 1). This pattern of streaking is of special advantage for visualizing the diffusion gradient of color inductors. When the Petri dishes were incubated at 25° C., color interaction between mutants was apparent within 36 hours of plating. The observations were recorded on the third and fourth days of incubation.

The results of plating two color variants at a time in all possible combinations are tabulated in Figure 2. Two mutants, O_1 and O_2, are excluded from this table, since they behaved the same as O_3 in all their reactions. Similar results were obtained on both the peptone medium and the synthetic ammonium-citrate medium, with the exception of retarded growth of variant P_{16} on the synthetic medium. No change in any of its interactions was found, however. In the combinations of reactors and inductors, the first appearance of the induced color can be noticed at the margins of the streak near the origin of the angular pattern. The color continues to deepen at this point and also develops inward and progresses along the margin of the streak (Fig. 1). If a thin streak (about 3 mm.) of the reactor is made parallel to the inductor, the entire reactor streak will exhibit the induction phenomenon. The normal HY strain does not act as an inductor with any of the

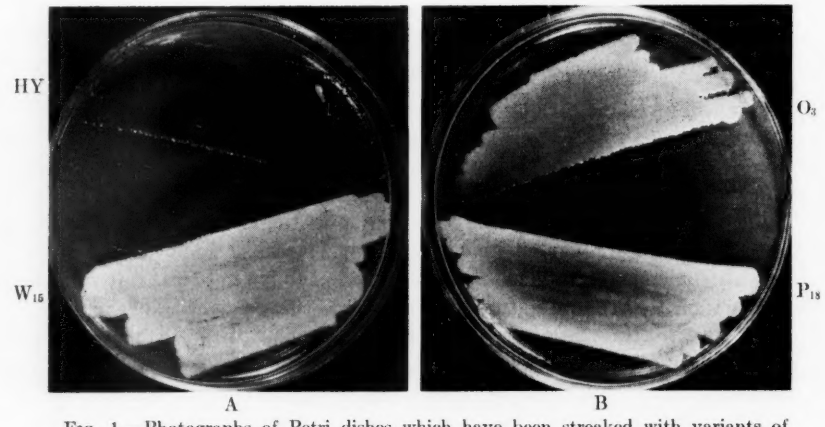

FIG. 1.—Photographs of Petri dishes which have been streaked with variants of *Serratia*. *A:* the normal HY strain and W_{15} do not exhibit any interaction. *B:* P_{18} is an inductor of red pigment in O_3. Note the color change of the O_3 streak in the region closest to P_{18}.

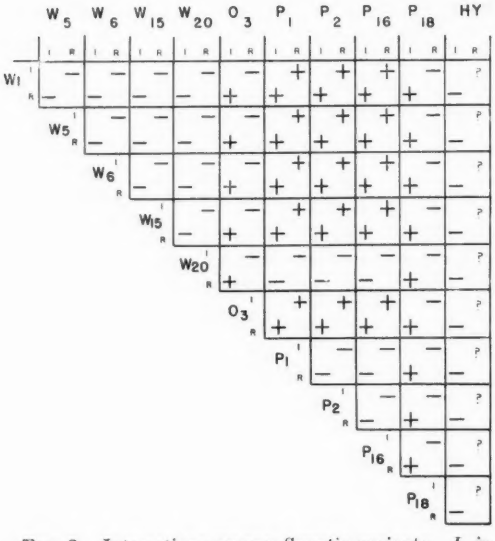

FIG. 2.—Interactions among *Serratia* variants. *I*, inductor; *R*, reactor. Read *I* of each column versus *R* of each row and *R* of each column versus *I* of each row. For example, the O_3 inductor causes a color change in W_1, which is labeled (+) as a reactor; but O_3 does not react (−) with the W_1 inductor. The last column is the wild-type HY strain.

mutants tested in the present survey; whether it is a reactor to any of the variants cannot be established, since the HY strain itself is deep red. Similarly, fourteen other variants (not included in the present report) were neither reactors nor inductors.

In Figure 2 interactions are listed as positive or negative only. However, the intensity of the color induced in various reactors differs. For example, P_{18}, which is a light-pink mutant, induces a red color resembling the HY pigment in the whites, W_1, W_5, W_6, W_{15}, but only a faint pink color appears in W_{20}. P_1 and P_2 are strong inductors for all the white variants except W_{20}, whereas the intensity of interaction of the white variants to the O_3 inductor can be expressed as $W_5 = W_6 > W_{15} > W_1 >>> W_{20}$.

When O_3 and W_5 are streaked in the pattern described above, the border of W_5 becomes pink. However, if O_3 is streaked within a millimeter of the white culture, then a stronger induction is indicated by the development of red pigment in W_5. These observations necessitate further qualitative and quantitative analyses of color interactions of the mutants of *Serratia*.

It is interesting to note that the normal HY strain produces a red color in the medium on aging. None of the mutants streaked beside the HY strain showed any color induction, even if the culture plates were incubated for several weeks. Color induction is a chemical interaction between the inductor and reactor substances and not merely the adsorption or accumulation of a pigment produced in the medium by the other variant on the same plate. This is also evident because white variants are capable of inducing further color in pink variants.

The present method provides a good opportunity for studying the biosynthesis of "prodigiosin" (tripyrrylmethene) and associated pigments of *Serratia*. Further, it may serve as a tool to differentiate the broad classes of white, pink, and orange variants by the utilization of their genotypic responses to different ecological conditions. For example, W_1 and W_{20} are morphologically indistinguishable, but the patterns of their responses to similar environmental conditions are quite distinct.

There is an exceptionally high frequency of mutations affecting the wild-type color of *Serratia marcescens*.[1, 2] In view of the nature of the reaction norms of the wild types of higher organisms, it seems that the entire system of pigmentation of *Serratia* is so delicately balanced and interwoven in the biological makeup of the organism that the mutation process can frequently influence the pattern of pigmentation, and there are few genetic modifiers, if any, to protect and insure the normal phenotype.

Summary.—Diffusible substances from some color variants of *Serratia marcescens* are capable of inducing pigment changes in other color variants. The interactions among twelve ultraviolet-induced variants as well as the normal HY strain have been described.

I would like to express my gratitude to Drs. D. Bonner and M. I. Bunting for the hospitality extended to me during the several weeks which I spent in their laboratories at the Department of Microbiology, Yale University. The facilities and equipment used during this visit were supplied through Atomic Energy Commission Grant No. AT(30-1)1273 to Dr. M . I. Bunting.

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INHERITANCE OF MATING TYPE AND A LETHAL FACTOR IN COSMARIUM BOTRYTIS VAR. SUBTUMIDUM WITTR.

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The wide diversity of cell shapes, ornamentations, and chloroplastids in the desmids, their unusual method of semicell regeneration in vegetative multiplication, and their rather large size have been of interest to investigators in many fields of experimental biology. Investigations have not been as fruitful as anticipated, perhaps because these organisms could not be induced to reproduce sexually in culture. The phenomenon of sexual reproduction is rarely observed in natural populations of desmids, and, although they have been cultivated successfully for many years,[1] it was not until recently[2] that strains were isolated which undergo sexual union in culture. The sexual strains of *Cosmarium botrytis* var. *subtumidum* have provided the first material for genetic investigations in the desmids.

The cells of *C. botrytis* var. *subtumidum* are haploid, and, inasmuch as the clonal cultures of the sexual strains[3] arose through multiplication from single cells, it may be assumed that the population in each culture is genetically pure. The strains multiply rapidly by vegetative multiplication in Pringsheim's soil-water medium[4] and can be maintained for months without transfer. Sexual reproduction is effected by mixing cells of the heterothallic strains in the small depressions of Pyrex spot plates or in shallow watch glasses. These are placed in deep Petri dishes containing water to prevent excessive evaporation from the depressions. Special gametes are not produced, the vegetative cells acting as such directly. Pairing of the gametes occurs within 24–48 hours after the strains have been mixed, and fusion of the gametic protoplasts is accomplished within another 24–48 hours. Actual fusion requires only four to seven minutes, but events leading up to fusion may occupy a much longer period of time. Details of this process have been reported more fully elsewhere.[2] Preliminary experiments have revealed that light is necessary for the initial pairing of the gametes, but the relation of the later phases of conjugation to light has not been ascertained.

Germination of the zygospore has been studied by DeBary,[5] Klebahn,[6] and more recently by the writer. At the beginning of germination, the protoplast escapes through a rupture in the walls of the zygospore. Nuclear and cytoplasmic divisions occur in the freed protoplast. Four nuclei are produced, but two disintegrate, resulting in the production of two rather than four gones by each germinating zygospore.

The two gones formed by a germinating zygospore were isolated, using fine glass pipettes, and were placed in separate tubes of soil-water culture medium. After sufficient vegetative multiplication had occurred, cells from each gonal culture were mixed separately with cells of each parental strain in order to ascertain their respective sexual potentialities. It had been suspected at the time of isolation of the original strains that determination of mating type might be due to more than a single factor, inasmuch as several strains of the same species from the same population had been found to be intersterile. It was of interest, therefore, to analyze the sister-gones from a single zygospore in order to ascertain whether a phenomenon such as the tetrapolar sexuality of certain fungi might exist and account for the apparently sterile strains in the natural population.

In matings of the heterothallic parental strains, occasional zygotes were observed which appeared to have died shortly after their formation, prior to the production of the spiny wall characteristic of the zygospores of the species. However, the abundance of normal zygospores produced at the same time indicated that such aborted zygotes probably resulted from some unfavorable situation of the environment. On the other hand, in certain backcrosses of gonal cultures with the *minus* parental strain, viable zygotes were never formed. Gametic pairing and fusion occurred as in those matings resulting in viable zygotes and zygospores, but soon after fusion, within 30 minutes to an hour when observed under the microscope, the zygotes lysed, and their contents escaped through small holes or ruptures in the membrane. The remains of the aborted zygote were in evidence for a long time as an accumulation of starch grains imbedded in the mucilaginous sheath which had been secreted earlier by the paired gametes. The empty walls of the gametes could be seen on each side of the starch mass.

Gonal cultures which were backcrossed with the *plus* parental strain never resulted in the production of aborted zygotes in numbers larger than could be accounted for by environmental effects; however, when these same cultures were crossed with other gonal cultures, a number of them failed to form viable zygotes. Table 1 summarizes the results of the various backcrosses.

Although it is generally assumed that meiosis occurrs in the germination of the zygospores of all placoderm desmids, it is only in *Hyalotheca dissiliens* (Sm.) Breb. that the process has been studied critically. In the zygospores of this alga, Pothoff[7] found that, as a result of two divisions, four haploid nuclei were formed. Three of these degenerated soon after division, the remaining one serving as the nucleus of the single gone produced by the germinating zygospore. In the genera *Closterium* and *Cosmarium*, whose zygospores commonly produce two gones, Klebahn[6] has shown that two nuclear divisions occur in the protoplast of the germinating zygospore, but he was not aware of the meiotic nature of the process. His figures clearly indicate, however, that, when each of the two nuclei formed by the first division undergoes a second division, a normal nucleus and a degenerative nucleus are the result. Preliminary cytological observations of the germinating zygospores of *Cosmarium botrytis* var. *subtumidum* indicate that the division of the nucleus is meiotic, approximately 30 bivalents being present at diakinesis. The writer's preparations confirm the observations of Klebahn that the two nuclei which survive are non-sister-nuclei of the four formed as a result of the two divisions.

In Table 1, the ratio of 56 to 49 for mating types is in close agreement with the expected ratio of 1 to 1, if mating type is determined by a single factor. In 22 of 23

TABLE 1

ANALYSIS FOR THE FACTORS OF MATING TYPE AND LETHALITY IN 105 GONES RESULTING FROM A CROSS BETWEEN THE *Plus* AND *Minus* PARENTAL STRAINS OF *Cosmarium botrytis* VAR. *subtumidum*

	NO. OF GONES
Total analyzed	105
Having *minus* mating type	56
Having *plus* mating type	49
Having *minus* mating type, resulting in viable zygotes when mated with *plus* parent	56
Having *minus* mating type, resulting in aborted zygotes when mated with *plus* parent	0
Having *plus* mating type, resulting in viable zygotes when mated with *minus* parent	25
Having *plus* mating type, resulting in aborted zygotes when mated with *minus* parent	24
Having *minus* mating type, resulting in viable zygotes when mated with *plus* gones known to carry lethals	36
Having *minus* mating type, resulting in aborted zygotes when mated with *plus* gones known to carry lethals	20

zygospores in which both gones were analyzed, the two gones were found to be of different mating types; in one instance, both gones were of the same mating type. Inasmuch as cytological preparations have indicated that the nuclei of the two gones are non–sister-nuclei of the second division, it is evident that the mating-type locus segregates, as a rule, at the first division. The one exception in which both gones were of the same mating type can best be explained by a crossover prior to the first division involving the mating-type locus and its centromere, with the chance survival of those two non–sister-nuclei which carried the same mating type. The probability of the survival of gones with the same mating type following such crossovers is 50 per cent. As in the ascus of *Neurospora* where the linear arrangement of the ascospores formed following meiosis forms an ordered tetrad indicating with great clarity the pattern of gene segregation, the disintegration of two non–sister-nuclei in the germinating zygospore of *Cosmarium* results in the production of two gones which may be analyzed in a similar manner. The loss of two nuclei is obviously a disadvantage in other respects.

In many haploid organisms, lethality is expressed by the inability to synthesize some organic substance necessary for a life-process, and, unless this substance is absorbed from an outside source, death results. These same lethals will usually express themselves in a homozygous dikaryon or diploid stage, but when they are associated with normal alleles they are usually masked by the action of the latter.

In *Cosmarium botrytis* var. *subtumidum* a lethal factor was present in a haploid clone (the *minus* parental strain) isolated from a natural population, but it did not produce any detectable effects in the haploid condition. It was not until after sexual fusion with a normal strain (the *plus* parental strain) and the resulting segregation and recombination at meiosis in the germinating zygospores that gones were produced which, when backcrossed with the lethal-containing parental strain, resulted in the combination of two lethal factors in the zygote and in its subsequent death. That the lethal factor does not express itself in the haploid cell seems to indicate that its action or lack of action probably is involved in biosyntheses essential only to the zygote and its maturation. Cytological preparations show that the fusion of the two gametic nuclei does not occur until long after formation of the resistant spiny walls of the zygospore. Therefore, it would appear that the lethal fac-

tor is expressed while the zygote is in a dikaryon stage, inasmuch as abortion occurs prior to the formation of the spines.

Analysis of the two gones from single zygospores indicated that, as a rule, one gone contains a lethal factor, while the other does not. In 21 of 23 zygospores analyzed this was found to be true. Of the remaining two, one zygospore produced two gones both of which carried lethals; the other produced two gones both of which carried the normal allele. These results indicate that the lethal factor is a single factor which segregates, as a rule, at the first nuclear division in the zygospore.

In Table 1 it can be seen that, of the 49 gones having the *plus* mating type, approximately 50 per cent show a recombination of the *plus*-mating-type locus with the lethal gene which was contributed by the *minus* parental strain. This is evident from the backcrosses which resulted in aborted zygotes. Such a percentage is to be expected if mating type and the lethal factors are determined by single genes located on different chromosomes. It should be noted that of the 56 gones having the *minus* mating type, only 20 possess lethal factors; 36 gones then represent recombinations of the *minus* mating type with the normal allele. This high percentage of recombination might be indicative of the greater viability of clones with the *minus* mating type and the normal allele in contrast to that of clones with the *minus* mating type and the lethal factor; but the relatively small number of progeny analyzed is hardly adequate for such conclusions at this time. It is very probable, however, that the genes for mating type and the lethal factor are unlinked.

Summary.—The heterothallic strains of *Cosmarium botrytis* var. *subtumidum* Wittr. provide the first material for genetic investigations in the desmids. As a result of the survival of only two non–sister-nuclei following the second division in meiosis, two gones rather than four are produced by a germinating zygospore.

The mating type of sexual strains is shown to be determined by a single gene, which segregates in the normal Mendelian fashion. The two mating types segregate, as a rule, in the first division of the meiotic process; therefore, the two gones from a single zygospore are usually of different mating types.

The abortion of all zygotes resulting from certain backcrosses of gones with the *minus* parental strain is due to a single factor which is undetectable in the haploid vegetative cell or in the heterozygous condition in the zygote. Zygotes formed by the fusion of two gametes, each carrying the lethal factor, always lyse shortly after their formation. The lethal factor is inherited as a single gene, segregating, as a rule, at the first division in meiosis. There appears to be no linkage between the genes for mating type and lethality.

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A FACTOR (OR MUTATOR GENE) INFLUENCING MUTATION RATES IN ESCHERICHIA COLI

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There is now considerable evidence, particularly in maize and in *Drosophila*, for the existence of factors which influence the rates of mutation of genes.[1, 2] The interpretations which have been offered are varied and involve, for example, mutator genes, heterochromatin, or even infective, virus-like particles. Although much still remains to be done, the study of these phenomena constitutes one approach to the general problem of the nature of gene action.

The present paper is concerned with the evidence for the existence of analogous effects in a microörganism, a variant of the well-known K12 strain of *Escherichia coli*.[3] Our recognition of the special properties of this bacterial variant is not without its instructive, even amusing aspects: in the course of an investigation on possible changes in the nutritive requirements of bacteria after selection for drug resistance, we had observed that a number of streptomycin-resistant K12 stocks acquired a need for one or more amino acids before growth ensued. In one series a requirement for phenylalanine was indicated for a number of independent isolates, in a frequency somewhat beyond that expected by chance. To investigate further this apparent association, we then determined the resistance of a stock phenylalanine-requiring K12 auxotroph which had not previously been in contact with streptomycin. At the inoculum dilution used, the culture appeared completely resistant to streptomycin, inocula of 0.1 ml. of full-grown culture growing out in subcultures containing 100 μg. or more streptomycin per milliliter, while other K12 stocks did not grow in drug concentrations beyond 5–10 μg. This appeared to be a verification of our working hypothesis concerning phenylalanine requirement and increased resistance to streptomycin.

Further investigation, with greater dilutions of the culture, revealed, however, that we were dealing not with a high level of resistance for the average organism in the culture but rather with an unusually high rate of mutation to resistance, which resulted in an exceptional number of resistant organisms in an otherwise susceptible population. Of perhaps more general interest is our later observation that, as in the *Drosophila* material, this increased mutation rate obtains toward a considerable variety of agents, although the mutation rate is not increased uniformly to all states and in fact appears to be normal in at least one instance. Finally, clear evidence has been obtained that the maintenance of this high rate of mutation is in no way related to a nutritional requirement for phenylalanine and that our original hypothesis in this regard, useful as it was in leading to further observations of interest, must be regarded as only a tentative working hypothesis, based on too small a series of initial observations and to be replaced in the light of additional experiments.

Cultures.—The relationships of the principal K12 cultures used, which are of importance for the argument below, are best understood from the following chart:[3]

K12 (Wild type, no amino acid requirement)
↓ X-ray-irradiated
58 (Biotin requirer)
↓ X-ray-irradiated
58-278 (Biotin + phenylalanine requirer, normal rate of mutation) — Several routine stock culture passages → 58–278 M*
↓ UV-irradiated
(Requirements as for 58-278, but is high mutating)
↓
Y24 (Biotin + phenylalanine + cystine requirer, normal rate of mutation)

NOTE: The designation M* will be applied below to the culture stocks which exhibit increased rates of mutation.

All the above organisms, except stock 58-278, were kindly supplied to us by our colleague, Dr. David Bonner. In the initial stage of this investigation, the fact that the unique properties of stock 58-278 M* were apparently acquired (by mutation?) during the culture passages was not appreciated, since the full chart had not been plotted. After the observation that stock Y24, which we had assumed to have been derived directly from stock 58-278 M* by further irradiation, did not possess exceptional mutating properties, the above chart was reconstructed from the records, and an effort was made to locate a sample of stock 58-278 which had not been passaged and which should therefore be more directly in the true line of descent than stock 58-278 M*. We were fortunate in that an original lyophilized sample of stock 58-278 meeting these requirements could be contributed by Professor E. L. Tatum, of Stanford University, who had preserved it from the above irradiation experiments, which he had conducted while still at Yale University. On test (see below), this culture was found to have only the normal, wild-type rate of mutation to streptomycin resistance. While this finding threw no light on the mechanism of origin of the high-mutating properties of stock 58-278 M* (except to suggest that they arose during the maintenance passages of the latter subline), it did account satisfactorily for the negative findings for stock Y24. As will be noted further below, the high-mutating properties, once introduced into a culture, are quite stable and persist even after further irradiation. Their absence from stock Y24 therefore required explanation.

Demonstration of Increased Numbers of Resistant Forms.—Evidence of resistant forms in an otherwise susceptible population may be provided by one of two types of experiments: titrations with varying inocula or direct plate counts on plain and on drug media.

In the former type of experiment it is common experience that, even with inocula as high as 1 ml. of full-grown culture, any of a variety of K12 or other coliform strains do not exhibit, more often than once in three samples of this size, a resistant organism capable of growing in 100 μg. of streptomycin per milliliter. The chance that an organism of this degree of resistance will be present decreases, of course, tenfold with each decimal dilution of the inoculum used, so that, with the inocula ordinarily employed in resistance tests (0.1 ml. or less), most cultures will be scored as "susceptible." In contrast, cultures of stock 58-278 M* invariably contain at least one resistant organism per hundredth of a milliliter. With less than this amount of inoculum, positive scores for resistance are obtained with decreasing frequency. On the other hand, in cultures of truly resistant organisms, each indi-

vidual microörganism possesses a considerable resistance, and practically the same dilution end-points are exhibited in plain and in drug-containing media. The behaviors of these three types of cultures, as estimated in part from other data given below, are illustrated in Table 1.

TABLE 1

PROBABILITIES OF POSITIVE SCORES FOR RESISTANCE, AS INFLUENCED BY INOCULUM SIZE, IN TUBE TITRATIONS OF K^{12} STRAINS

(Screening Concentration = 100 μg. Streptomycin per Milliliter)

INOCULUM SIZE (ML.)	STRAIN EMPLOYED		
	"Normal" Wild or 58-278	"High-mutating," 58-278 M*	"Fully Resistant," Selected from 58-278 M* or Non-M*
1.0	+/3	+	+
0.1	+/30	+	+
0.01	+/300	+	+
0.001	+/3,000	+/5	+
0.0001	+/30,000	+/50	+

NOTE: + = growth in drug, with consequence positive "score" for resistance of culture; +/3 = positive an average of only once in 3 trials; +/30 = positive an average of only once in 30 trials, etc.
The fully resistant strains are derived in the usual manner, by an initial isolation on streptomycin agar.

More quantitative estimates are afforded by platings on solid media (Fig. 1). The various non-M* cultures, when plated directly, without preselection for resistance, give counts which decrease rapidly with increases in the drug concentration (curve *A*). A similar type of behavior has been recorded for other organisms and antibiotics.[4] A fully resistant culture, on the other hand, gives a quantitative recovery over this range (curve *B*). The M* culture, although following the general course of curve *A* down to about 0.0001 per cent of the initial population, then diverges significantly from this distribution because of the appreciable number of more highly resistant organisms which are present (curve *C*).

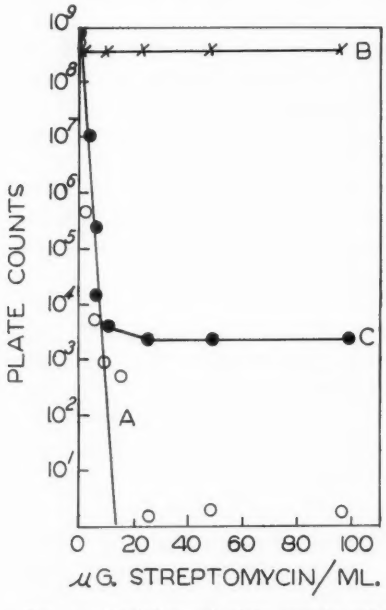

FIG. 1.—Variation of plate counts with drug concentration. Curve *A* (*open circles*): typical K12 culture. Resistant forms may or may not appear, depending on sample variations. Curve *B:* culture fully resistant to to streptomycin. Curve *C* (*dark circles*): high-mutating culture.

Random Origin of Resistant Forms.—In a typical experiment a culture of stock 58-278 M* was diluted to give about one hundred organisms per inoculum, as checked by plate counts. Since the ratio of susceptible to resistant organisms is of the order of 10^6, an inoculum of this size is very unlikely to contain a "preformed" resistant organism. Portions of this inoculum were then distributed into each of nine broth tubes, which were incubated to full density, after which the total

number of organisms and the percentage of resistant forms in each tube were determined. Each value recorded in Table 2 is the average of two plate counts. The large variance for the counts on drug agar, in contrast to the small variance for the total count, in these determinations, for a series of cultures originating from common small inocula, may be taken as evidence for the random origin of the resistant forms.[5, 6]

TABLE 2

MEANS AND VARIANCES FOR COUNTS OF TOTAL AND RESISTANT ORGANISMS IN SERIES OF CULTURES FROM COMMON SMALL INOCULA

Tube No.	Total Count ($\times 10^2$)	Resistant Count ($\times 10$)	$\frac{\text{Total Count}}{\text{Resistant Count}} \times 10^6$
1	94	150	0.63
2	74	83	0.89
3	108	100	1.08
4	112	68	1.65
5	99	204	0.49
6	101	126	0.80
7	98	290	0.34
8	139	123	1.12
9	87	57	1.52
Mean	101	133	0.95
Variance	323	5,465	19.7
Var./mean	3.2	41	21

In a variation of this experiment, a culture of 58-278 M* was plated on brain heart agar, and, after incubation, eight typical colonies were picked and each resuspended in separate 0.5-ml. portions of water. These inocula were then each diluted serially, by tenfold steps, through sets of broth tubes. After the latter had been incubated, the last tube in each set which showed growth was identified. It could be assumed, from the manner of preparation, that each represented a culture begun with ten, or less, organisms. These eight secondary cultures were then plated (in appropriate dilutions) on plain agar and on agar containing 100 μg. streptomycin per milliliter. The statistical measures obtained were as follows: total count, mean = 4.7×10^3, with variance 1.7 times the mean; resistant count, 2.8×10^3, with variance 20.1 times the mean. This difference in the variance ratios leads to the same conclusion as the previous experiment.

Mutation Rates to Streptomycin Resistance.—These were determined from the number of tubes which contained no resistant variants.[5] The inocula used varied from 10 to 500 organisms in the different experiments; they were checked by plate counts in each instance.

For the determination of the mutation rates of all the "normal" comparison cultures, 1.0 ml. of undiluted brain heart broth was used for each tube in the set. For the M* cultures the broth was diluted 1:300 before inoculation, so as to reduce the final density; without this step, the density in each tube would be such as to result in a hundred or more resistant organisms, and the invariable positive scores would preclude quantitative evaluation of the rate.

Following incubation to final density, all tubes were diluted with 5–10 volumes of broth containing streptomycin; those failing to show secondary growth after reincubation were scored as negative. When difficulty was experienced in reading,

the contents of a tube were streaked on streptomycin agar for verification of the absence of resistant forms. The rates determined are tabulated in Table 3; that for the "high-mutating" or M* strain is seen to be about one hundred times the "normal" rate.

TABLE 3

MUTATION RATES TO STREPTOMYCIN RESISTANCE OF "NORMAL" AND "HIGH-MUTATING" K12 STRAINS OF *E.coli*, AS ESTIMATED BY THE NEGATIVE TUBE METHOD

CULTURE (WITH AUXOTROPHIC REQUIREMENT)	FINAL SCREENING CONCENTRATION OF STREPTOMYCIN (μG/ML)	INDIVIDUAL VALUES OBSERVED	MEAN	STANDARD DEVIATION
	High-mutating Strain; All Rates Are to Be Multiplied by 10^{-8}			
58-278 M* (biotin-, phenylalanine-, large colony form)	100	5.0, 2.0, 1.5, 7.0, 3.4, 3.6	3.8	1.8
	Normal Comparison Strains; All Rates Are to Be Multiplied by 10^{-10}			
58-278 B (small non-M* colonies derived from 58-278 M* above)	500	7.3, 6.3, 2.1, 2.7, 3.0, 2.5, 6.6, 4.1, 4.1, 2.2, 3.7, 8.6	4.4	2.2
58-278 (biotin-, phenylalanine-)	100	3.6	3.6	...
Biotin-, methionine-	500	3.2, 1.6, 2.3, 3.0, 5.5, 5.0, 2.2, 1.5	3.0	1.6
Wild type K12	500	6.7, 3.1, 3.0, 1.4, 8.7, 2.6, 2.3	4.0	2.7

All determinations were made at 37° C., in brain heart broth of pH 7.4. Each individual value tabulated is from an experiment employing 50–100 tubes. The significance of the large and small colony forms is explained in the text.

Streptomycin Dependency.—It is general experience that there is a marked strain influence on the percentage of streptomycin-resistant forms isolated which are also streptomycin-dependent. For some coliform organisms it may run as high as 40–50 per cent; for K12 wild type we have found about 25 per cent of dependent forms. The low rate of isolation of dependent forms from the M* cultures is especially noteworthy; for 58-278 M* it is only 0.5 per cent. These latter dependent forms are also atypical in that, once isolated, they exhibit an extraordinary tendency toward reversion to streptomycin independency, presumably due to the close association of the M* factor (or gene) with this locus. As an example, in one experiment 1 independent form was secured for each 420 streptomycin-dependent organisms plated, while for the wild type this ratio is increased to 1 in 10^7 plated.

Separation of High-mutational Property and Nutritional Requirement.—The invalidity of our original suggestion concerning an association between streptomycin resistance and a requirement for phenylalanine was evident from the data for cultures Y24 and 58-278 (see chart above). This was confirmed by analysis of a series of auxographs derived from *E. coli*, strain W-NZ, kindly furnished us by Dr. Bernard Davis. Each of these cultures exhibited a requirement for phenylalanine (with or without additional amino acid requirements), but none had any unusual resistance or mutational characteristics.

Although the evidence appeared conclusive that a requirement for either biotin or phenylalanine was not invariably, or even frequently, associated with the initiation of a high mutational rate, there still remained the possibility that these particular requirements were in some way essential for its maintenance, once it had been established. The invalidity of this second hypothesis could readily be proved

by recombination experiments, through which the auxotrophic requirements could be removed without disturbing the high-mutational characteristics.

For these, the original procedure of Tatum and Lederberg[3] was followed. The first cross effected was as shown below:

Biotin-, phenylalanine-, M* (58-278 M*) × Threonine-, leucine-, non-M*
↓ Recombination conditions, with final screening on minimal agar
24 prototrophs, all M*

This cross has since been repeated in a number of experiments, with yields of hundreds of prototrophs of similar characteristics. In addition, it is possible by irradiation of the prototrophs to introduce new auxotrophic markers without disturbance of the M* characteristic. When some of these new auxotrophs are recombined with certain other non-M* cultures, not all the resulting recombinants are found to possess the M* characteristic. This behavior, which will be reported in more detail elsewhere, is not, however, necessarily in conflict with other data reported here on the stability of the M* characteristic but may merely reflect differences in segregation, such as have been reported for other crosses.[7, 8]

Mention should be made here of another observation made early which is of some interest. When the original culture of 58-278 M* was received, it was plated on agar and found to give rise to both small and large colonies. The high-mutational characteristics and the ability to recombine were limited to the latter colonies, which then constituted our stock 58-278 M* for subsequent experiments. The small colonies, designated 58-278 B (Table 3), not only possessed the normal mutational rate but, unlike culture 58-278 itself, gave no prototrophs in recombination experiments. This observation, which was anomalous at the time, may now be viewed in the light of recent work on strain polarity.[7, 8] Experiments to test this are now in progress.

Specificity of the High-mutational Effect.—A survey was made of seventy-eight cultures isolated as *E. coli* in the clinical laboratory of our hospital, utilizing as screening technique any unusual numbers of resistant organisms which appeared after plating on streptomycin agar (100 μg. per milliliter). Some twenty such cultures yielded significant numbers of colonies, although most yielded none at all. As might be expected from the procedure used, most of these high positive scores were due to sampling variations, in which there was by chance a resistant mutant initially present or a mutation had occurred early during growth, and on replication of these with larger numbers of independent culture tubes no unusual number of resistant organisms appeared. Two stocks did, however, give positive scores on this confirmatory test, and, although one gave variable results on further repetitions, it is likely that one or both of these represent cultures which have mutation rates to streptomycin resistance significantly above the average. Unfortunately, these cultures were discarded before the more general properties of stock 58-278 M* were ascertained, and it therefore cannot be stated whether or not the high mutational rate of these other coliforms is limited to streptomycin resistance.

Although the present report deals largely with the latter characteristic, as has just been suggested the unusual features of stock 58-278 M* embrace a wider range. Thus it appears from preliminary experiments involving platings on agar containing various agents that from ten to several hundred times as many colonies appear

from the M* stocks as arise from the normal comparison strains. Included in this respect are resistances to diverse agents, including chloramphenicol, sodium fluoride, or certain T phages, such as T1, T4, T5, or T3h. Mutations to abilities (or reversals from abilities) to ferment certain sugars, particularly xylose, also appear to be increased. Significantly, however, the rate of mutation to resistance to phage T2 appears to be entirely in the "normal" range, as judged by comparisons with stock 58-278 and the wild type. This is the sole exception so far encountered

The further quantitation of most of these processes is entirely feasible, and experiments to this end are now under way. The possibility of differential selective effects[9] may make simple colony comparisons unreliable, however, and the more laborious mutation-rate determinations must ultimately be made, as has been done above for streptomycin resistance. With proper design, such data should be independent of any selective forces which might bias the rate determinations.

The fact that selective effects alone are not responsible for the larger number of mutants observed in the M* stocks, as contrasted with the non-M* stocks, could readily be demonstrated by specially designed reconstruction experiments. In these, inocula of a resistant stock which contained either 0.5–2.0 or 5–20 organisms were introduced into tubes containing 10^9 "susceptibles" of stock 58-278, and streptomycin was then added. In all instances resistant organisms were recovered in the same frequency as for inocula of the resistant stocks alone, in plain broth. In its preparation this resistant stock, derived from culture 58-278, had been given only one purification transfer in streptomycin, so as to militate against any selective forces, except for resistance, which would differentiate it from its parental stock. We may therefore conclude that in the present instance even a very small number of resistant variants derived from stock 58-278 can grow out in the presence of a large number of "sensitives," and hence the lower mutation rate for this non-M* culture is not due to a masking effect, or to false negatives. Although care was taken to have the resistant organism as homologous to the parental culture as possible, this experimental approach does not guarantee that there may not be present other types of potentially resistant organisms which cannot compete at all with the "sensitives" even to the degree of occasional growth necessary for isolation. It may only be added that additional attempts to detect their presence by marked dilution of the culture[9] were unsuccessful.

Implications.—Although, as cited earlier, the properties of mutator genes in higher forms have been described by numerous investigators, detailed descriptions of their occurrence in bacteria appear to be lacking. Evidence for varying rates of acquisition of drug resistance,[10] or for reversions of alleles at a single locus (in *Neurospora*[11]) has, however, been published.

Cultures of exceptional mutation rate require special recognition in the design of clinical tests for resistance, which must score a culture as either "resistant" or "susceptible," at some preassigned drug level. The danger of false scoring for resistance would appear to be overcome by employment of two amounts of inoculum, one low and one high, much as in the test for potential penicillinase producers.

Although the numbers of resistant forms produced may be sufficient to effect, with some tests, a scoring as a "resistant" culture, they may not be numerous enough to affect the clinical course under chemotherapy, in situations in which cellular or humoral immunity can dispose of the majority of the organisms present. On the

other hand, where the clinical situation demands that all organisms be checked by chemotherapy alone, the high rate of production of resistant forms may have serious consequences, even though the laboratory test indicates that the bulk of the culture population is sensitive.

The potentialities for the use of the high-mutating factor, here tentatively considered to be a mutator gene, in the exploration of problems in microbial genetics have scarcely been realized. Fortunately for laboratory work, this characteristic appears to be quite stable and has been preserved during dozens of culture transfers over a period of three years; this is in marked contrast to the lability of some mutator genes in higher forms.[1]

The nonuniformity of the increases in mutation rates to different states, apparently the result of mediation by the mutator gene, is a matter of some interest. Further quantitation is, however, needed before valid comparisons with linkage data and map distances can be made. The zero effect recorded in the case of mutation to resistance to phage T2 is of especial interest here; in higher forms there is evidence that mutator genes may influence the mutation of genes located in different chromosomes.[1, 2]

The unequivocal interpretation of quantitative data for M* and non-M* comparison strains is not simple and often involves additional information which is not readily available. Thus, even if differences in the mutation rates appear for some phenotypic changes, it is not always readily ascertainable whether these are due to comparable genic changes or whether there may not be factors other than the mere presence or absence of the mutator gene which are quantitatively influencing the observed rates. Experiments recognizing this difficulty are now under way and, if successful, should permit a more exact delineation of the mutator gene properties in *E. coli*, strain K12.

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EQUATIONS IN FINITE FIELDS

By N. C. Ankeny*

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Communicated by H. S. Vandiver, Sept. 10, 1954

Consider the monomial equation

$$a_0 x_0^{m_0} + a_1 x_1^{m_1} + \ldots + a_r x_r^{m_r} = 0 \tag{1}$$

in a finite field. The number of solutions of such equations has recently stimulated a great deal of work by such mathematicians as H. Hasse,[1] L. K. Hua,[2] H. Davenport, H. S. Vandiver, and A. Weil.[3] Weil has recently made certain conjectures about the number of solutions of such equations as (1) and also more generally about the number of rational points on varieties without singular points.

First we shall deal with the case where (1) is an equation in a field p elements. where p is a prime. Consider equation (1) as a congruence (mod p); m_j divides $p - 1$; m_j is fixed; and $a_0, \ldots, a_r$ are fixed nonzero rational integers.

Denote by $N(a_0, a_1, \ldots, a_r, m_0, \ldots, m_r, p) = N(p)$ the number of solutions of equation (1) (mod p). One can then prove Theorem I.

THEOREM I. *There exists a constant A which depends only upon $m_0, \ldots, m_r$ such that*

$$N(p) = p^r + A\theta_p p^{(r+1)/2},$$

where $|\theta_p| < 1$ for all primes p, and $\overline{\lim}_{p \to \infty} |\theta_p| = 1$.

One can further prove that $|\theta_p|$ is everywhere dense in the unit interval. In fact, given any two real numbers $0 \leq \alpha < \beta \leq 1$, denote by $\mathfrak{D}(U, \alpha, \beta)$ the number of primes less than U for which $\alpha < |\theta_p| < \beta$. Then $\mathfrak{D}(U, \alpha, \beta) \sim c(\alpha, \beta)\ \mathrm{U}(\log \mathrm{U})^{-1}$ as $U \to +\infty$, where $c(\alpha, \beta)$ depends only upon α, β. It might be suspected that $c(\alpha, \beta)$ depends only upon the difference $\beta - \alpha$, but this is not true. The relationship is a good deal more complex.

Another way of considering equation (1) is for the case where $a_0, a_1, \ldots, a_r$ are any elements in a field with p elements; m_j divides $p - 1$, m_j fixed; and $(a_j/p)_{m_j} = l^{(2\pi i/m)l_j}$, where l_j are fixed rational integers for $j = 0, 1, \ldots, r$.

Denote $N'(l_0, l_1, \ldots, l_r, p) = N'(p)$ the number of solutions of equation (1). One can then prove Theorem II.

THEOREM II. *There exists a constant A' which depends only upon $l_0, \ldots, l_r$, such that*

$$N'(p) = p^r + A'\varphi_p p^{r/2},$$

where $|\varphi_p| < 1$ for all primes p, and $\overline{\lim}_{p \to \infty} |\varphi_p| = 1$.

The remarks following Theorem I about θ_p are all applicable to φ_p.

One can prove that $A' \leqq \prod_{j=0}^{\gamma} (m_j - 1)$, but in general the inequality must hold. One can compute the value of A' for every $l_0, \ldots, l_r$, but this is quite complicated.

Both Theorems I and II can be generalized to finite fields with p^ν elements, with certain corresponding changes in the definitions.

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MONOTONE APPROXIMATION IN DYNAMIC PROGRAMMING AND THE CALCULUS OF VARIATIONS

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1. *Introduction.*—In a previous note[1] we outlined some applications of the functional equation approach of the theory of dynamic programming[2, 3] to the characterization of extremal curves and eigenvalues in the calculus of variations. A more detailed account of this formalism will be found in other papers by the author.[4, 5]

The purpose of the present note is to show that another concept in the theory of dynamic programming, that of "approximation in policy space," may also be utilized to yield some interesting results in the calculus of variations. As we shall indicate below, this concept leads to the solution of variational problems by iterative techniques which yield monotone approximation, and indeed monotone convergence, as we shall show elsewhere.

To illustrate this new concept, we shall first consider a discrete dynamic programming problem and then present the analogous treatment of a continuous version, which leads to the problem of maximizing the integral $J(y) = \int_0^T F(x, y, t)\, dt$, subject to the relations $\dot{x} = G(x, y, t)$, $x(0) = c$. Following this, we shall discuss the application of this technique to the eigenvalue problem associated with the equation $u'' + \lambda^2 \phi(t) u = 0$, $u(0) = u(1) = 0$.

2. *Monotone Convergence in Dynamic Programming.*—Let us consider the functional equation

$$f(x) = \max_{0 \le y \le x} [g(y) + h(x - y) + f(ay + b(x - y))] = T(f), \qquad (2.1)$$

where $0 < a, b < 1$, which arises in connection with various types of multistage allocation processes.[3] It is readily shown that if $g(x)$ and $h(x)$ are continuous in x over an interval $[0, c]$, with $g(0) = h(0) = 0$, then, starting with any initial function $f_0(x)$ which is continuous over $[0, c]$ and zero at $x = 0$, the iterative procedure $f_{n+1}(x) = T(f_n)$ yields the unique solution of equation (2.1) which is continuous at $x = 0$.

To obtain monotone convergence, we approximate first in policy space rather than function space. A policy is, with reference to equation (2.1) a choice of $y = y(x)$ with $0 \le y(x) \le x$. Let $y_0 = y_0(x)$ be an initial policy, and let $f_0(x)$ be computed by recurrence from the functional equation

$$f_0(x) = g(y_0) + h(x - y_0) + f_0(ay_0 + b(x - y_0)), \qquad f_0(0) = 0, \tag{2.2}$$

which is to say $f_0(x)$ is the return obtained using the policy $y_0(x)$. It is immediately clear that $f_1(x)$ as determined by

$$f_1(x) = T(f_0) \tag{2.3}$$

is greater than or equal to $f_0(x)$ for all $x \geq 0$. From this it follows inductively that $f_{n+1}(x) = T(f_n)$ is greater than or equal to $f_n(x)$ for all $x \geq 0$. Hence we have monotone convergence.[6]

3. *Monotone Approximation in the Maximization of* $\int_0^T F(x, y, t)\, dt$.—As in earlier papers,[1, 5] we write

$$f(a, c, T) = \operatorname*{Max}_y \int_a^{a+T} F(x, y, t)\, dt, \tag{3.1}$$

where $\dot{x} = G(x, y, t)$, $x(a) = c$. The function f satisfies the functional equation

$$f_T = \operatorname*{Max}_v [F(c, v, a) + G(c, v, a) f_c + f_a]. \tag{3.2}$$

As in previous notes, we shall avoid for the moment all discussion of necessary or sufficient conditions for these equations to hold and shall present only the basic formalism.

An approximation in policy space is now a choice of y as a function of x, a, and T, which is to say $v = v(a)$ as a function of c, a, and T. Let v_0 represent some such initial choice, and let $f_0(a, c, T)$ denote the function obtained in this way. Then $f_0(a, c, T) = \int_a^{a+T} F(x_0, y_0, t)\, dt$, $x_0 = G(x_0, y_0, t)$, $x_0(a) = c$, and f_0 satisfies the partial differential equation

$$f_{0T} = F(c, v_0, a) + G(c, v_0, a) f_{0c} + f_{0a}, \qquad f_0(a, c, 0) \equiv 0. \tag{3.3}$$

A further approximation, v_1, to an optimal policy is now determined by the condition that v_1 maximize the function of v given by

$$H(v, f_0) = F(c, v, a) + G(c, v, a) f_{0c} + f_{0a}. \tag{3.4}$$

Let f_1 be the function determined by v_1, satisfying the equation $f_1 = H(v_1, f_1)$. We now determine a second approximation, v_2, by the condition that it maximize $H(v, f_1)$ and continue in this fashion, obtaining a sequence of functions $\{v_n\}$ and $\{f_n\}$.

Let us now demonstrate the essential result that the sequence $\{f_n\}$ is monotone increasing in n for all a, c and $T \geq 0$. We have

$$\begin{aligned} f_{1T} - f_{0T} &= H(v_1, f_1) - H(v_0, f_0) \\ &= H(v_1, f_0) - H(v_0, f_0) + H(v_1, f_1) - H(v_1, f_0) \end{aligned} \tag{3.5}$$

or

$$(f_1 - f_0)_T = A(c, T, a) + (f_1 - f_0)_c B(c, T, a) + (f_1 - f_0)_a, \tag{3.6}$$

where, from the manner in which v_1 was determined, $A \geq 0$.

From this, and the boundary conditions at $T = 0$ for f_0 and f_1, it follows readily that $f_1 - f_0 \geq 0$ for all a, c, and T. The same argument shows that $f_{n+1} \geq f_n$.

4. *Sketch of a Convergence Proof.*—The above argument yields monotone approximation without discussing the question of convergence. One approach to the convergence proof is through the consideration of the corresponding discrete problem, which yields the functional equation

$$f(a, c, N + 1) = \operatorname*{Max}_{y} [F(c, y, a) + f(a + 1, c + G(c, y, a), N)], \quad (4.1)$$

where $f(a, c, N) = \operatorname*{Max}_{y} J(y) = \operatorname*{Max}_{y} \sum_{k=a}^{N} F(x_k, y_k, k)$, subject to $x_{k+1} - x_k = G(x_k, y_k, k)$, $x_a = c$.

5. *Monotone Convergence in Eigenvalue Problems.*—The problem of determining the values of λ^2 which permit nontrivial solutions of

$$u'' + \lambda^2 \phi(t) u = 0, \qquad u(0) = u(1) = 0, \quad (5.1)$$

is, under light restrictions upon $\phi(t)$, equivalent to the problem of determining the relative maxima of $\int_0^1 \phi(t) u^2 \, dt$ subject to the constraints $\int_0^1 u'^2 \, dt = 1$, $u(0) = u(1) = 0$. To attack the problem by means of the functional equation technique used above, we consider the more general problem of determining the maximum of

$$J(u) = \int_a^1 \phi(t) u^2 \, dt + k \int_a^1 (1 - t) u \phi(t) \, dt, \quad (5.2)$$

subject to the same constraints. Setting $f(a, k) = \operatorname{Max} J(u)$, we obtain[1, 4] the equation

$$f_a = \operatorname*{Min}_{w} \left[\left(f - \frac{k f_k}{2} \right) w^2 - w \left(\frac{2 + k\psi(a)}{1 - a} \right) \right], \quad (5.3)$$

where $\psi(a) = \int_a^1 (1 - t)^2 \phi(t) \, dt$ and $w = u'(a)$. A choice of a policy is a choice of $w = w(a, k)$. The method of successive approximations used above may again be employed, with the proof of monotonicity as above in essential details.

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EQUATIONS IN FINITE FIELDS

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$$a_0 x_0^{m_0} + a_1 x_1^{m_1} + \ldots + a_r x_r^{m_r} = 0 \tag{1}$$

in a finite field. The number of solutions of such equations has recently stimulated a great deal of work by such mathematicians as H. Hasse,[1] L. K. Hua,[2] H. Davenport, H. S. Vandiver, and A. Weil.[3] Weil has recently made certain conjectures about the number of solutions of such equations as (1) and also more generally about the number of rational points on varieties without singular points.

First we shall deal with the case where (1) is an equation in a field p elements. where p is a prime. Consider equation (1) as a congruence (mod p); m_j divides $p - 1$; m_j is fixed; and $a_0, \ldots, a_r$ are fixed nonzero rational integers.

Denote by $N(a_0, a_1, \ldots, a_r, m_0, \ldots, m_r, p) = N(p)$ the number of solutions of equation (1) (mod p). One can then prove Theorem I.

THEOREM I. *There exists a constant A which depends only upon $m_0, \ldots, m_r$ such that*

$$N(p) = p^r + A\theta_p p^{(r+1)/2},$$

where $|\theta_p| < 1$ for all primes p, and $\overline{\lim}_{p \to \infty} |\theta_p| = 1$.

One can further prove that $|\theta_p|$ is everywhere dense in the unit interval. In fact, given any two real numbers $0 \leq \alpha < \beta \leq 1$, denote by $\mathfrak{D}(U, \alpha, \beta)$ the number of primes less than U for which $\alpha < |\theta_p| < \beta$. Then $\mathfrak{D}(U, \alpha, \beta) \sim c(\alpha, \beta)\ \mathrm{U}(\log \mathrm{U})^{-1}$ as $U \to +\infty$, where $c(\alpha, \beta)$ depends only upon α, β. It might be suspected that $c(\alpha, \beta)$ depends only upon the difference $\beta - \alpha$, but this is not true. The relationship is a good deal more complex.

Another way of considering equation (1) is for the case where $a_0, a_1, \ldots, a_r$ are any elements in a field with p elements; m_j divides $p - 1$, m_j fixed; and $(a_j/p)_{m_j} = l^{(2\pi i/m)l_j}$, where l_j are fixed rational integers for $j = 0, 1, \ldots, r$.

Denote $N'(l_0, l_1, \ldots, l_r, p) = N'(p)$ the number of solutions of equation (1). One can then prove Theorem II.

THEOREM II. *There exists a constant A' which depends only upon $l_0, \ldots, l_r$, such that*

$$N'(p) = p^r + A'\varphi_p p^{r/2},$$

where $|\varphi_p| < 1$ for all primes p, and $\overline{\lim}_{p \to \infty} |\varphi_p| = 1$.

The remarks following Theorem I about θ_p are all applicable to φ_p.

One can prove that $A' \leq \prod_{j=0}^{\gamma} (m_j - 1)$, but in general the inequality must hold. One can compute the value of A' for every $l_0, \ldots, l_r$, but this is quite complicated.

Both Theorems I and II can be generalized to finite fields with p^ν elements, with certain corresponding changes in the definitions.

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[1] H. Davenport and H. Hasse, *J. reine angew. Math.*, **172**, 151–182, 1935.

[2] L. K. Hua and H. S. Vandiver, these PROCEEDINGS, **34**, 258–263, 1948; L. K. Hua and H. S. Vandiver, *ibid.*, **35**, 94–99, 1949.

[3] A. Weil, *Bull. Am. Math. Soc.*, **55**, 497–588, 1949.

MONOTONE APPROXIMATION IN DYNAMIC PROGRAMMING AND THE CALCULUS OF VARIATIONS

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1. *Introduction.*—In a previous note[1] we outlined some applications of the functional equation approach of the theory of dynamic programming[2, 3] to the characterization of extremal curves and eigenvalues in the calculus of variations. A more detailed account of this formalism will be found in other papers by the author.[4, 5]

The purpose of the present note is to show that another concept in the theory of dynamic programming, that of "approximation in policy space," may also be utilized to yield some interesting results in the calculus of variations. As we shall indicate below, this concept leads to the solution of variational problems by iterative techniques which yield monotone approximation, and indeed monotone convergence, as we shall show elsewhere.

To illustrate this new concept, we shall first consider a discrete dynamic programming problem and then present the analogous treatment of a continuous version, which leads to the problem of maximizing the integral $J(y) = \int_0^T F(x, y, t)\, dt$, subject to the relations $\dot{x} = G(x, y, t)$, $x(0) = c$. Following this, we shall discuss the application of this technique to the eigenvalue problem associated with the equation $u'' + \lambda^2 \phi(t) u = 0$, $u(0) = u(1) = 0$.

2. *Monotone Convergence in Dynamic Programming.*—Let us consider the functional equation

$$f(x) = \max_{0 \le y \le x} [g(y) + h(x - y) + f(ay + b(x - y))] = T(f), \qquad (2.1)$$

where $0 < a, b < 1$, which arises in connection with various types of multistage allocation processes.[3] It is readily shown that if $g(x)$ and $h(x)$ are continuous in x over an interval $[0, c]$, with $g(0) = h(0) = 0$, then, starting with any initial function $f_0(x)$ which is continuous over $[0, c]$ and zero at $x = 0$, the iterative procedure $f_{n+1}(x) = T(f_n)$ yields the unique solution of equation (2.1) which is continuous at $x = 0$.

To obtain monotone convergence, we approximate first in policy space rather than function space. A policy is, with reference to equation (2.1) a choice of $y = y(x)$ with $0 \le y(x) \le x$. Let $y_0 = y_0(x)$ be an initial policy, and let $f_0(x)$ be computed by recurrence from the functional equation

$$f_0(x) = g(y_0) + h(x - y_0) + f_0(ay_0 + b(x - y_0)), \qquad f_0(0) = 0, \tag{2.2}$$

which is to say $f_0(x)$ is the return obtained using the policy $y_0(x)$. It is immediately clear that $f_1(x)$ as determined by

$$f_1(x) = T(f_0) \tag{2.3}$$

is greater than or equal to $f_0(x)$ for all $x \geq 0$. From this it follows inductively that $f_{n+1}(x) = T(f_n)$ is greater than or equal to $f_n(x)$ for all $x \geq 0$. Hence we have monotone convergence.[6]

3. *Monotone Approximation in the Maximization of* $\int_0^T F(x, y, t)\, dt$.—As in earlier papers,[1, 5] we write

$$f(a, c, T) = \max_y \int_a^{a+T} F(x, y, t)\, dt, \tag{3.1}$$

where $\dot{x} = G(x, y, t)$, $x(a) = c$. The function f satisfies the functional equation

$$f_T = \max_v [F(c, v, a) + G(c, v, a) f_c + f_a]. \tag{3.2}$$

As in previous notes, we shall avoid for the moment all discussion of necessary or sufficient conditions for these equations to hold and shall present only the basic formalism.

An approximation in policy space is now a choice of y as a function of x, a, and T, which is to say $v = v(a)$ as a function of c, a, and T. Let v_0 represent some such initial choice, and let $f_0(a, c, T)$ denote the function obtained in this way. Then $f_0(a, c, T) = \int_a^{a+T} F(x_0, y_0, t)\, dt$, $x_0 = G(x_0, y_0, t)$, $x_0(a) = c$, and f_0 satisfies the partial differential equation

$$f_{0T} = F(c, v_0, a) + G(c, v_0, a) f_{0c} + f_{0a}, \qquad f_0(a, c, 0) \equiv 0. \tag{3.3}$$

A further approximation, v_1, to an optimal policy is now determined by the condition that v_1 maximize the function of v given by

$$H(v, f_0) = F(c, v, a) + G(c, v, a) f_{0c} + f_{0a}. \tag{3.4}$$

Let f_1 be the function determined by v_1, satisfying the equation $f_1 = H(v_1, f_1)$. We now determine a second approximation, v_2, by the condition that it maximize $H(v, f_1)$ and continue in this fashion, obtaining a sequence of functions $\{v_n\}$ and $\{f_n\}$.

Let us now demonstrate the essential result that the sequence $\{f_n\}$ is monotone increasing in n for all a, c and $T \geq 0$. We have

$$\begin{aligned} f_{1T} - f_{0T} &= H(v_1, f_1) - H(v_0, f_0) \\ &= H(v_1, f_0) - H(v_0, f_0) + H(v_1, f_1) - H(v_1, f_0) \end{aligned} \tag{3.5}$$

or

$$(f_1 - f_0)_T = A(c, T, a) + (f_1 - f_0)_c B(c, T, a) + (f_1 - f_0)_a, \tag{3.6}$$

where, from the manner in which v_1 was determined, $A \geq 0$.

From this, and the boundary conditions at $T = 0$ for f_0 and f_1, it follows readily that $f_1 - f_0 \geq 0$ for all a, c, and T. The same argument shows that $f_{n+1} \geq f_n$.

4. *Sketch of a Convergence Proof.*—The above argument yields monotone approximation without discussing the question of convergence. One approach to the convergence proof is through the consideration of the corresponding discrete problem, which yields the functional equation

$$f(a, c, N + 1) = \operatorname*{Max}_{y} [F(c, y, a) + f(a + 1, c + G(c, y, a), N)], \quad (4.1)$$

where $f(a, c, N) = \operatorname*{Max}_{y} J(y) = \operatorname*{Max}_{y} \sum_{k=a}^{N} F(x_k, y_k, k)$, subject to $x_{k+1} - x_k = G(x_k, y_k, k)$, $x_a = c$.

5. *Monotone Convergence in Eigenvalue Problems.*—The problem of determining the values of λ^2 which permit nontrivial solutions of

$$u'' + \lambda^2 \phi(t) u = 0, \qquad u(0) = u(1) = 0, \quad (5.1)$$

is, under light restrictions upon $\phi(t)$, equivalent to the problem of determining the relative maxima of $\int_0^1 \phi(t) u^2 \, dt$ subject to the constraints $\int_0^1 u'^2 \, dt = 1$, $u(0) = u(1) = 0$. To attack the problem by means of the functional equation technique used above, we consider the more general problem of determining the maximum of

$$J(u) = \int_a^1 \phi(t) u^2 \, dt + k \int_a^1 (1 - t) u \phi(t) \, dt, \quad (5.2)$$

subject to the same constraints. Setting $f(a, k) = \operatorname{Max} J(u)$, we obtain[1, 4] the equation

$$f_a = \operatorname*{Min}_{w} \left[\left(f - \frac{k f_k}{2} \right) w^2 - w \left(\frac{2 + k\psi(a)}{1 - a} \right) \right], \quad (5.3)$$

where $\psi(a) = \int_a^1 (1 - t)^2 \phi(t) \, dt$ and $w = u'(a)$. A choice of a policy is a choice of $w = w(a, k)$. The method of successive approximations used above may again be employed, with the proof of monotonicity as above in essential details.

[1] R. Bellman, "Dynamic Programming and a New Formalism in the Calculus of Variations," these PROCEEDINGS, **40**, 231–235, 1954.

[2] See R. Bellman, *An Introduction to the Theory of Dynamic Programming* (RAND Corporation Report No. R-245, 1953).

[3] See R. Bellman, "Some Problems in the Theory of Dynamic Programming," *Econometrica*, January, 1954.

[4] R. Bellman, "Dynamic Programming and the Calculus of Variations. I" (RAND Corporation Paper No. P-495, 1954).

[5] R. Bellman, "Dynamic Programming and the Calculus of Variations. II" (RAND Corporation Paper No. P-512, 1954).

[6] R. Bellman, "Computational Problems in the Theory of Dynamic Programming" (RAND Corporation Paper No. P-423, presented to the Symposium on Applied Mathematics, Santa Monica, 1954).

REPRESENTATIONS OF SEMISIMPLE LIE GROUPS. V

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Let G be a connected semisimple Lie group and $\mathfrak{g}_0$ its Lie algebra over the field R of real numbers. Let $x \to Ad(x)$ denote the adjoint representation of G. A maximal connected abelian subgroup A of G is called a Cartan subgroup if $\mathfrak{g}_0$ is fully reducible under $Ad(A)$. The corresponding subalgebra of $\mathfrak{g}_0$ is called a Cartan subalgebra. Two Cartan subgroups A_1, A_2 are called conjugate (in G) if $A_2 = xA_1x^{-1}$ for some $x \in G$. Experience shows[1] that there is a close connection between the classes of conjugate Cartan subgroups and the various series of irreducible unitary representations of G which appear in the reduction of its regular representation. The object of this note is to give a general method of constructing irreducible unitary representations of G from each such class.

Let $X \to adX$ denote the adjoint representation $\mathfrak{g}_0$. Put $B(X,Y) = \text{sp}(adXadY)$. $(X, Y \in \mathfrak{g}_0)$. A subalgebra $\mathfrak{l}_0$ of $\mathfrak{g}_0$ is called compact if the quadratic form $B(X, X)$ is negative definite on $\mathfrak{l}_0$. Let $\mathfrak{k}_0$ be a maximal compact subalgebra of $\mathfrak{g}_0$. We denote by $\mathfrak{p}_0$ the subspace of $\mathfrak{g}_0$ orthogonal to $\mathfrak{k}_0$ under the bilinear form $B(X, Y)$. Then $\mathfrak{g}_0$ is the direct sum of $\mathfrak{k}_0$ and $\mathfrak{p}_0$. Let $\mathfrak{h}_0$ be a Cartan subalgebra of $\mathfrak{g}_0$. It is possible to choose $\mathfrak{k}_0$ in such a way that $\mathfrak{h}_0 = \mathfrak{h}_{\mathfrak{p}_0} + \mathfrak{h}_{\mathfrak{k}_0}$, where $\mathfrak{h}_{\mathfrak{p}_0} = \mathfrak{h}_0 \cap \mathfrak{h}_{\mathfrak{p}_0}$, $\mathfrak{h}_{\mathfrak{k}_0} = \mathfrak{h}_0 \cap \mathfrak{k}_0$. Let $H_1, \ldots, H_p$ be a base for $\mathfrak{h}_{\mathfrak{p}_0}$ over R. We order real linear functions on $\mathfrak{h}_{\mathfrak{p}_0}$ lexicographically with respect to this base. For any such function λ let $\mathfrak{g}_{0,\lambda}$ denote the set of all $X \in \mathfrak{g}_0$ such that $[H, X] = \lambda(H)X$ $(H \in \mathfrak{h}_{\mathfrak{p}_0})$. Then $\mathfrak{n}_0 = \sum_{\lambda > 0} \mathfrak{g}_{0,\lambda}$ is a nilpotent subalgebra of $\mathfrak{g}_0$. Let $\mathfrak{m}_0$ denote the set of all $X \in \mathfrak{g}_0$ such that $[X, H] = 0$ and $B(X, H) = 0$ for all $H \in \mathfrak{h}_{\mathfrak{p}_0}$. Then $\mathfrak{m}_0$ is a reductive[2] subalgebra of $\mathfrak{g}_0$, and $\mathfrak{h}_{\mathfrak{k}_0}$ is a maximal abelian subalgebra of $\mathfrak{m}_0$.

Let K, A_+, M_0, N be the analytic subgroups of G corresponding to $\mathfrak{k}_0$, $\mathfrak{h}_{\mathfrak{p}_0}$, $\mathfrak{m}_0$, and $\mathfrak{n}_0$. Let M_k be the centralizer and M'_k the normalizer of A_+ in K. Then $W = M'_k/M_k$ is a finite group. Put $M = M_kM_0$ and $S = MA_+N$. Then M and S are closed subgroups of G, and we have the following generalization of a lemma of F. Bruhat:[3]

Lemma 1. *There exist only a finite number of distinct double cosets SxS $(x \in G)$.*

In case $\mathfrak{h}_{\mathfrak{c}_0}$ is a maximal abelian subspace of $\mathfrak{p}_0$, these double cosets are in a natural 1-1 correspondence with the elements of W.

Let λ_0 be an irreducible unitary representation of M on a Hilbert space U and ξ a (unitary) character of A_+. If we put $\lambda(man) = \lambda_0(m)\xi(a)$ $(m \in M, a \in A_+, n \in N)$, λ defines a representation of S. Let π be the induced[4] representation of G corresponding to λ, and let $\mathfrak{H}$ denote the representation space of π. If Z is the center of G, $Z \subset M$, and therefore both λ and π map Z into scalars. Let Ω denote the set of all equivalence classes of finite-dimensional irreducible representations of K, and let $\mathfrak{H}_{\mathfrak{D}}$ $(\mathfrak{D} \in \Omega)$ be the set of all elements of $\mathfrak{H}$ which transform under $\pi(K)$ according to $\mathfrak{D}$.

Theorem 1. *π is a unitary quasi-simple[5] representation of G, and dim $\mathfrak{H}_{\mathfrak{D}} < \infty$ for every $\mathfrak{D} \in \Omega$.*

It follows from this theorem that π decomposes into a (discrete) direct sum of at most a countable number of irreducible unitary representations. However, in view of Lemma 1 and Bruhat's results,[6] it seems likely that π is irreducible at least "in general." Since M/M_0Z is finite, the above procedure will be applicable as soon as we have a method of constructing irreducible unitary representations of M_0. Such a method is given in the following note.[7]

Let $x \to x^*$ denote the natural mapping of G onto $G^* = G/Z$, and let dx^* denote the Haar measure on G^*. Let π be a unitary irreducible representation of G on a Hilbert space $\mathfrak{H}$. Then for any fixed ψ in $\mathfrak{H}$, $|(\psi, \pi(x)\psi)|$ depends only on x^*. We say that π is square-integrable if $\int_{G^*}|(\psi, \pi(x)\psi)|^2\, dx^* < \infty$ for every $\psi \in \mathfrak{H}$. One can prove that this is indeed the case if there exist a finite number of elements ϕ_i, ψ_i $(1 \leq i \leq r)$ in $\mathfrak{H}$ such that the function $f(x) = \sum_{i=1}^{r} (\phi_i, \pi(x)\psi_i)$ is not identically zero and $\int_{G^*}|f(x)|^2 dx^* < \infty$. The following theorem gives the analogue of the Schur orthogonality relations for square-integrable representations.[8]

THEOREM 2. *Let π and π' be two irreducible unitary representations of G on the Hilbert space $\mathfrak{H}$ and $\mathfrak{H}'$, respectively. Suppose that they are both square-integrable and that they both define the same character of Z. Then*

$$\int_{G^*}\overline{(\phi, \pi(x)\psi)}(\phi', \pi'(x)\psi')\, dx^* = 0 \qquad (\phi, \psi \in \mathfrak{H}, \qquad \phi', \psi' \in \mathfrak{H}'),$$

unless π and π' are equivalent. On the other hand, if U is a unitary mapping of $\mathfrak{H}$ on $\mathfrak{H}'$ under which π and π' are equivalent, then

$$\int_{G^*}\overline{(\phi, \pi(x)\psi)}\ (\phi', \pi'(x)\psi')\, dx^* = c(\phi', U\phi)\ (U\psi, \psi'),$$

where c is a positive real number independent of ϕ, ψ, ϕ', ψ'.

We shall give a general method of constructing square-integrable representations in the following note.[7]

[1] See V. Bargmann, *Ann. Math.*, **48,** 568–640, 1947; Harish-Chandra, these PROCEEDINGS, **38,** 337–342, 1952; and I. M. Gelfand and M. I. Graev, *Doklady Akad. Nauk S.S.S.R.*, N.S., **92,** 221–224, 1953.

[2] See L. Koszul, *Bull. Soc. Math. France,* **78, 22,** 1950.

[3] F. Bruhat, *Compt. rend. Acad. sci.* (Paris), **238,** 550–553, 1954.

[4] G. W. Mackey, *Ann. Math.*, **55,** 106, 1952.

[5] See Harish-Chandra, these PROCEEDINGS, **37,** 170–173, 1951.

[6] F. Bruhat, *Compt. rend. Acad. sci.* (Paris), **238,** 38–40, 1954.

[7] These PROCEEDINGS, **40,** 1078–1080, 1954.

[8] See Bargmann, *op. cit.*, p. 634. R. Godement (*Compt. rend. Acad. sci.* (Paris), **225,** 657–659, 1947) has proved a similar result for any locally compact unimodular group. However, in case the center of G is infinite, his theorem does not seem to include ours.

REPRESENTATIONS OF SEMISIMPLE LIE GROUPS. VI

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We keep to the notation of the preceding note.[1] Since $\mathfrak{m}_0$ is reductive, there will be no essential loss of generality from the point of view of irreducible unitary representations of M_0 if we assume that $\mathfrak{m}_0$ is semisimple. Then $\mathfrak{m}_0 \cap \mathfrak{k}_0$ is a maximal compact subalgebra of $\mathfrak{m}_0$, and $\mathfrak{h}_{\mathfrak{k}_0}$ is a Cartan subalgebra of $\mathfrak{m}_0$. Moreover, $\mathfrak{m}_0 = \mathfrak{m}_0 \cap \mathfrak{k}_0 + \mathfrak{m}_0 \cap \mathfrak{p}_0$. Our problem of constructing irreducible unitary representations of M_0 is therefore the same as that for G, under the additional assumption that a maximal abelian subalgebra of $\mathfrak{k}_0$ is also maximal abelian in $\mathfrak{g}_0$. Hence we shall now assume that $\mathfrak{h}_0 \subset \mathfrak{k}_0$. Let C be the field of complex numbers and $\mathfrak{g}$ the complexification of $\mathfrak{g}_0$. We denote by $\mathfrak{h}$, $\mathfrak{k}$, and $\mathfrak{p}$ the subspaces of $\mathfrak{g}$ spanned over C by $\mathfrak{h}_0$, $\mathfrak{k}_0$, and $\mathfrak{p}_0$, respectively. For any root α of $\mathfrak{g}$ (with respect to $\mathfrak{h}$), choose an element $X_\alpha \neq 0$ in $\mathfrak{g}$ such that $[H, X_\alpha] = \alpha(H)X_\alpha$ $(H \in \mathfrak{h})$. Then X_α is unique apart from a factor in C, and it lies either in $\mathfrak{k}$ or in $\mathfrak{p}$. We call α a compact root if $X_\alpha \in \mathfrak{k}$. Either α and $-\alpha$ are both compact, or they are both noncompact.

Let $\mathfrak{B}$ be the universal enveloping algebra of $\mathfrak{g}$ and $\mathfrak{X}$ the subalgebra of $\mathfrak{B}$ generated by $(1, \mathfrak{k})$. Let π be a representation of $\mathfrak{B}$ on a vector space V. We shall say that π has an extreme vector if there exists an element $\psi \neq 0$ in V and a linear function Λ on $\mathfrak{h}$ such that the following conditions hold:

1. For every root α at least one of the two vectors $\pi(X_\alpha)\psi$, $\pi(X_{-\alpha})\psi$ is zero.
2. $\pi(H)\psi = \Lambda(H)\psi$ for all $H \in \mathfrak{h}$, and dim $\pi(\mathfrak{X})\psi$ is finite.

ψ is then called an extreme vector belonging to the weight Λ. Now, if ψ is an extreme vector, it is possible to define a lexicographic order (with respect to a suitable base on $(-1)^{1/2}\mathfrak{h}_0$ over R) in the space of all linear functions on $\mathfrak{h}$ which take real values on $(-1)^{1/2}\mathfrak{h}_0$, such that[2] $\pi(X_\alpha)\psi = 0$ for every root $\alpha > 0$. From now on we shall keep this order fixed and say that an extreme vector ψ is positive if $\pi(X_\alpha)\psi = 0$ for all $\alpha > 0$. Put $H_\alpha = [X_\alpha, X_{-\alpha}]$ and $\epsilon_\alpha = 1$ or -1 according as α is compact or not. Then it is possible to select X_α in such a way that $\alpha(H_\alpha) = 2$ and $(-1)^{1/2}(X_\alpha + \epsilon_\alpha X_{-\alpha})$, $(X_\alpha - \epsilon_\alpha X_{-\alpha})$ are both in $\mathfrak{g}_0$ for every root α. Let $\alpha_1, \ldots, \alpha_r$ be all the positive compact roots. We say that a root β is totally positive if every root of the form $\beta + m_1\alpha_1 + \ldots + m_r\alpha_r$ (where $m_1, \ldots, m_r$ are integers) is positive. Clearly, if β is totally positive, it is positive and noncompact. Let Q be the set of all totally positive roots and Q' the remaining set of positive roots.

Theorem 1. *Let π be an irreducible representation of $\mathfrak{B}$ on V with a positive extreme vector ψ belonging to the weight Λ. Then, if $\alpha \in Q'$, $\lambda_\alpha = \Lambda(H_\alpha)$ is a nonnegative integer and $\pi(X_{-\alpha}^{\lambda_\alpha + 1})\psi = 0$. Moreover, if Q is empty, dim V is finite.*

Conversely, suppose that we are given a linear function Λ on $\mathfrak{h}$ such that $\Lambda(H_\alpha)$ is a nonnegative integer for every $\alpha \in Q'$. Then it can be shown that there exists an irreducible representation π of $\mathfrak{B}$ on a vector space V with a positive extreme vector ψ belonging to the weight Λ, and π is unique up to equivalence. Furthermore, assuming that G is simply connected, we can find an irreducible quasisimple[3] representation σ of G on a Hilbert space $\mathfrak{H}$ and an element $\phi \in \mathfrak{H}$ which is well behaved[3]

under σ such that the representation of $\mathfrak{B}$ defined on $\mathfrak{H}_0 = \sigma(\mathfrak{B})\phi$ is equivalent to π under the mapping $\sigma(b)\phi \leftrightarrow \pi(b)\psi$ $(b \in \mathfrak{B})$. In order that π be infinitesimally unitary,[4] it is necessary that $\Lambda(H_\beta)$ be real and ≤ 0 for all positive noncompact roots β.

If $\mathfrak{g}_0$ is simple, only the two following cases are possible:

1. There are no totally positive roots.
2. Every positive noncompact root is totally positive.

The second case can occur only if the center of $\mathfrak{k}_0$ is $\neq \{0\}$. Moreover, if $\mathfrak{g}_0$ is not compact, the representation π of Theorem 1 can never be infinitesimally unitary unless we are in case 2. So now let us assume that $\mathfrak{g}_0$ is not compact and we are in case 2. Then we can select a fundamental system of positive roots $\alpha_0, \alpha_1, \ldots, \alpha_l$ such that α_0 is noncompact, while α_i $(1 \leq i \leq l)$ are compact. A linear function Λ on $\mathfrak{h}$ fulfils the condition of Theorem 1 if and only if $\Lambda(H_{\alpha_i})$ $(1 \leq i \leq l)$ are nonnegative integers. We denote by π_Λ the corresponding representation of $\mathfrak{B}$. We have seen that π_Λ cannot be infinitesimally unitary unless $\Lambda(H_{\alpha_0})$ is real and ≤ 0. On the other hand, it can be shown that, if λ_i $(1 \leq i \leq l)$ are given nonnegative integers, there exists a real number $\lambda_0 \leq 0$ such that π_Λ is infinitesimally unitary whenever $\Lambda(H_{\alpha_i}) = \lambda_i$ $(1 \leq i \leq l)$ and $\Lambda(H_{\alpha_0}) \leq \lambda_0$.

Let us now suppose that G (which is again semisimple) has a faithful finite-dimensional representation.[5] Then there exists a complex analytic group G_c with the Lie algebra $\mathfrak{g}$, which contains G as the (real) analytic subgroup corresponding to $\mathfrak{g}_0$. Let $\mathfrak{n} = \sum_{\alpha > 0} CX_\alpha$, and let A_c and N_c be the complex analytic subgroups of G_c corresponding to $\mathfrak{h}$ and $\mathfrak{n}$, respectively. Then $G_c^0 = GA_cN_c$ is an open connected subset, and therefore a complex submanifold, of G_c. Let ξ be a complex analytic character of A_c. We consider the space $\mathfrak{H}_\xi$ of all holomorphic functions ϕ on G_c^0 such that $\phi(xan) = \phi(x)\xi(a)$ $(x \in G_c^0, a \in A_c, n \in N_c)$, and

$$||\phi||^2 = \int_G |\phi(x)|^2 \, dx < \infty.$$

(Here dx is the Haar measure of G.) It is not difficult to see[6] that $\mathfrak{H}_\xi$ is complete with respect to the above norm, and so it is a Hilbert space. If $\mathfrak{H}_\xi \neq \{0\}$, we can define a unitary representation π_ξ of G on it by the rule $(\pi_\xi(x)\,\phi)(y) = \phi\,(x^{-1}y)$ $(x \in G, y \in G_c^0)$. It can be proved that this representation is irreducible and square-integrable.[7] Now assume again that G is simple and not compact. Then $\mathfrak{H}_\xi = \{0\}$ unless we are in case 2. So now let us suppose that we are in case 2. Given two complex analytic characters ξ and ξ' of A_c, we shall say that $\xi' \leq \xi$ if $\xi'(\exp H_{\alpha_i}) = \xi(\exp H_{\alpha_i})$ $(1 \leq i \leq l)$ and $|\xi'(\exp H_{\alpha_0})| \leq |\xi(\exp H_{\alpha_0})|$. Again $\mathfrak{H}_\xi = \{0\}$, unless $|\xi(\exp H_{\alpha_i})| \geq 1$ $(1 \leq i \leq l)$. On the other hand, if ξ_0 is any (complex analytic) character of A_c such that $|\xi_0(\exp H_{\alpha_i})| \geq 1$ $(1 \leq i \leq l)$, we can find a character $\xi_1 \leq \xi_0$ such that $\mathfrak{H}_\xi \neq \{0\}$ if $\xi \leq \xi_1$. If Λ is a linear function on $\mathfrak{h}$ such that $\xi(\exp H) = e^{\Lambda(H)}$ $(H \in \mathfrak{h})$ and $\mathfrak{H}_\xi \neq \{0\}$, the corresponding representation of $\mathfrak{B}$ defined under π_ξ is equivalent to π_Λ.

[1] These PROCEEDINGS, **40**, 1076–1077, 1954.

[2] This result has also been obtained independently by A. Borel.

[3] See these PROCEEDINGS, **37**, 170–173, 1951.

[4] See *Trans. Am. Math. Soc.*, **75**, 233, 1953.

[5] It is possible to avoid this assumption, which is made here only for simplicity.

[6] See S. Bochner and W. T. Martin, *Several Complex Variables* (Princeton, N.J.: Princeton University Press, 1948), p. 117.

[7] Our procedure for constructing π_ξ is a generalization of the method used by Bargmann (*Ann. Math.*, **48**, 620, 1947) and Gelfand and Graev (*Izvest. Akad. Nauk, S.S.S.R.* **17**, 189–248, 1953) in certain special cases.

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*SPONTANEOUS TESTICULAR TERATOMAS IN AN INBRED STRAIN OF MICE**

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Descriptions of teratoid tumors of the mammalian testis, other than for man and the horse, are rare. In mice teratoma testis has not heretofore been reported. Furthermore, there are only a few descriptions of the ovarian counterpart of this type of tumor in the mouse.[1, 2, 3, 4] In view of the paucity of information concerning teratomas in mice, their occurrence in the testes of an inbred strain is of considerable interest, especially since they appear spontaneously in a definite proportion of males.

The testicular teratomas to be described here are not simple malformations but, like the ovarian lesions mentioned above, are true tumors which possess the power of progressive growth. They have all appeared in strain 129 mice and are composed of a variety of embryonic and adult tissues which are not normally found in the testis. Anatomical studies of teratoma testis in the horse and in man revealed that, although they are typically disorganized, certain tissue correlations may exist.[5] Little is known of the functional capacities of teratomatous tissues, such as secretion hemopoiesis, movement, and nervous activity.

Experimental investigations of teratomas have been conspicuously few. Testicular teratomas have been produced experimentally in fowl by intratesticular injection of inorganic zinc or copper salts.[6] Twombley, Meisel, and Strout[7] found that intrasplenic grafts of day-old rat testes in castrate animals became tumorous. In most cases Leydig cell tumors developed, but a few of the grafts resembled teratomas in that they contained striated muscle and fat. However, Firminger[8] has pointed out that "the finding of striated muscle and fat would be adequately explained on the basis of inclusion of peritesticular tissue in the transplants." Day-old mouse testes similarly grafted to the spleens of castrate hosts have not developed tumors of any type.[9]

A variety of transplantation experiments have been performed, using ovarian teratomas of the mouse.[2, 4] Following repeated serial transplantations, these tumors have retained their pleomorphic character. Pluripotent embryonic cells appear to give rise to both rapidly differentiating cells and others which, like themselves, remain undifferentiated. It seems clear from these studies that embryonic-type cells which do not undergo adult-type histogenesis are responsible for progressive growth.

Despite several attempts to gain information leading to a better understanding of teratomas, there is little conclusive evidence to elucidate such basic problems as their origin, etiological factors which influence their incidence, the developmental stage during which they arise, and the sequence of tissue differentiation during the development of the tumors.

The main difficulties in studying teratomas of large mammals are associated with the rarity and large size of the tumors, the length of life of the host, and the long latent period between incipient tumorigenesis and clinical recognition of the tumor. These difficulties may to a degree be surmounted by examination of the gonads of a large number of individuals of an inbred strain of mice in which there is a high incidence of spontaneous testicular teratomas.

INCIDENCE OF TESTICULAR TERATOMAS IN STRAIN 129 MICE

Thus far, 32 spontaneous testicular teratomas have been found in our colony of strain 129 mice. Ten additional cases have been observed in strain 129 mice from another colony from which ours was derived. In order to determine the incidence of these tumors, an account has been kept of the number and age of male mice examined. This information was not available for all 42 teratomas. Of 3,557 males, 30, or nearly 1 per cent, have borne these neoplasms.

There seems to be no particular age group in which these tumors predominantly occur, although our data may be inconclusive because the number of males examined at the age extremes has been relatively small (see Table 1). The youngest mouse

TABLE 1

INCIDENCE OF SPONTANEOUS TESTICULAR TERATOMAS ACCORDING TO AGE

Age Group	No. Males Examined	No. Teratomas		Per Cent Teratomas
0–20 days	390	7		1.8
20–30 days	949	7		0.74
Over 30 days	257	2		0.73
Total	1,596	16	Mean	1.0

in which a testicular teratoma was observed at autopsy was 8 days of age; the oldest, 210 days. We are currently examining serial sections of day-old strain 129 testes in an attempt to determine whether or not these teratomas are congenital.

There is no indication that the incidence varies with the age of the mother, the litter size, or the litter seriation (see Table 2). Of forty cases where laterality is

TABLE 2

A LIST OF TESTICULAR TERATOMAS GIVING THE AGE OF THE MOUSE BEARING THE TUMOR, THE AGE OF THE MOTHER, THE LITTER SIZE, AND THE LITTER SERIATION

Mouse No.	Age of Mouse (Days)	Age of Mother (Months)	Litter Size	Litter Seriation
LS 28	25	7	7	3
144	21	4	5	1
176	25	2	8	1
202	20	4	1	3
215	210	4	4	1
233	66	5	4	3
305	18	6	2	5
308	13	8	4	8
186	23	6	?	1
335	31	7	6	3
369	24	6	5	4
391	37	5	?	2

known, 30 teratomas occurred in the left testis. So far, they have occurred bilaterally in only one case.

Ovaries of strain 129 female mice have been examined without finding teratomas. However, in one 6-month-old female, $F_1129 \times DBA$, a large teratoma similar in structure to those described by Jackson and Brues[2] and Fekete and Ferrigno[4] was found in the left ovary. Examination of the gonads of other inbred strains of mice in our colony has not revealed teratomas.

In about 5 per cent of strain 129 males, one testis is reduced in size. About 80 per cent of these small testes occur on the left side. In most cases, a congenital hematoma in the testes is accompanied by degeneration of all or a fraction of the spermatogenic tubules. Examination of such small testes has failed to reveal teratomatous growths.

DESCRIPTION OF STRAIN 129 TESTICULAR TERATOMAS

Usually there are no external symptoms indicating a teratomatous testis. Most have been discovered during autopsy. In five of the forty-two cases, however, there has been pronounced enlargement of the affected gonad, such as the one shown in Figure 1. In these large growths, dark hemorrhagic patches are apparent through

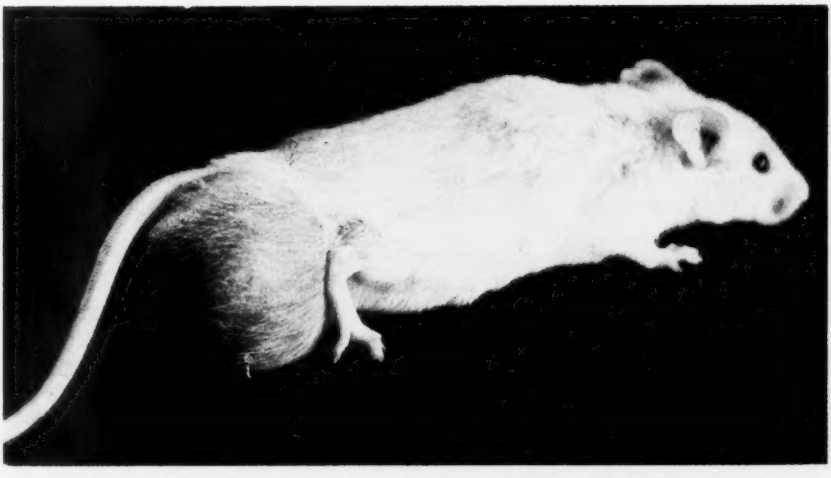

FIG. 1.—Spontaneous testicular teratoma in a 6-month-old strain 129 mouse.

the scrotum. When extirpated, the smaller tumors usually appear as solid masses comprising one-fourth to three-fourths of the testicular volume. Usually the masses inclose cysts filled with clear or bloody fluid. Many of the teratomas we have examined have contained palpable nodules of bone and cartilage. Occasionally fat may be identified grossly, but the remainder of the tissues appears as a soft, opaque mass. When teratomas are found, some are fixed *in toto*, while others are bisected, half to be used for transplantation and half for histological study.

Histologically, the tissue components of most of the teratomas vary only slightly. Most commonly present are nervous tissue, epithelia of various types, cartilage,

bone with well-differentiated marrow, muscle, fat, and glandular tissue, in that order (see Fig. 2). Undifferentiated embryonic cells are not ubiquitous in highly

FIG. 2.—Section of a strain 129 testis, showing spermatogenic tubules and a teratoma containing striated muscle, cartilage, bone, nervous tissue, and cysts lined with cuboidal epithelium.

differentiated teratomas. We have not found organoids, such as teeth and skin, which are commonly found in teratomas of man and horses. Seminomatous and trophoblastic elements and structures resembling early embryos have not been observed in any of the tumors examined.

There seems to be a correlation between the size of a teratoma and the number of tissue types it contains (see Table 3). Similarly, there is an apparent relationship between the age of the animal bearing the tumor and the variety of tissue types in the teratoma. In four of the younger animals (from 8 to 16 days), only nervous tissue, cuboidal epithelium, and undifferentiated embryonic tissue were present.

TABLE 3

TISSUE TYPES OBSERVED IN TUMORS OF VARIOUS SIZES

Size* of Tumor	No. Tumors	Nervous Tissue	Cuboidal Epith.	Cartilage and Bone	Goblet Cells	Muscle	Fat	Stratified Epith.	Glandular Tissue
1/4	4	4	4	2	0	0	0	0	0
1/2	4	4	4	2	2	3	1	2	0
3/4	9	9	9	8	8	9	6	5	6

* Size of tumor expressed as fraction of testicular volume.

The tumor of a fifth animal in this age group, however, contained representatives of several additional tissue types. Clusters of dividing spermatocytes have been frequently observed interspersed among nerve cells. Apparently the teratomas infiltrate the spermatogenic tubules.

EXPERIMENTAL

1. *Tumor Transplantation.*—Several of the teratomas have been minced and grafted subcutaneously via trocar into weaning-age strain 129 hosts. Of fifteen such grafts of spontaneous teratomas, all have persisted in intact and castrate animals of both sexes, but to date only one has developed into a rapidly growing transplantable tumor.

The rapidly growing transplantable tumor was derived from a highly differentiated growth in the testis of a 40-day-old mouse and is currently in the sixteenth generation of serial transplantation. It is composed of prolific undifferentiated cells, many of which are polyploid and show mitotic irregularities. The tumor is retransplanted every two weeks, when it reaches about 2 cm. in diameter. The structure as well as the growth rate of the tumor has remained unchanged since the initial transplant generation. When homogenized in saline and injected intraperitoneally, this transplantable tumor has flourished in the ascitic fluid of strain 129 mice and has persisted as a transplantable ascitic tumor.

At the time of the sixth transplant generation the tumor was grafted into five DBA/1 hosts. In three cases the tumor grew in the foreign strain and has survived serial transplantation in that strain for twelve generations. Grafts into four other foreign strains have succumbed to the homograft reaction within about 10 days.

In contrast, all other grafts of spontaneous teratomas have not increased in size after periods as long as 9 months but have persisted as small hard nodules of well-differentiated adult-type tissues. These grafts are similar in growth characteristics and histological composition to homogenized 13–14-day fetal tissue injected subcutaneously.

2. *Testicular Transplantation.*—In none of six cases in which day-old strain 129 testes were implanted into the spleens of castrate hosts for periods of 5–12 months did testicular tumors develop. The grafts resembled cryptorchid testes. These data are being supplemented.

DISCUSSION

The strain 129 mice of our colony possess a genetic makeup predisposing testicular elements toward the formation of teratomas. The mechanisms involved are as yet obscure, even as to the organ or tissue initially affected. The effect may not necessarily be confined to gonadal components but may include the environment of the developing gonad.

As to the etiology of these tumors, two features might lead us to discount either a hypothesis which restricts consideration to testicular tissues or a hypothesis which implies a general physiological influence, such as hormone unbalance: (1) Seventy-five per cent of the forty teratomas have been found in the left testis. It might be expected that a hereditary condition which affects particular gonadal cells would do so as frequently on one side as on the other. Similarly, a generalized physiological influence would be likely to affect both sides with equal frequency. It may be significant in this respect that the developing ovary of young strain 129 mice does not undergo spontaneous tumorigenesis. The development of gonadal teratomas in strain 129 mice seems to depend upon a sex difference. It is possible that a difference such as gonadal migration may be involved. In this connection, it may be well to point out that a difference in the time of migration exists between the right and the left testes. (2) In about 5 per cent of the males of strain 129 mice, one testis, usually the left, shows degenerative changes and is reduced in size. Examination of such small testes has failed to reveal teratomatous growths. Here, again, however, a hereditary tendency with a primarily unilateral effect suggests the likelihood that the two conditions, small testis and teratoma testis, may have etiological factors in common.

The amount of the testis damaged in these small testes varies from slight to total. It is conceivable that slight damage, not enough to cause degeneration of the testis, may predispose it toward teratogenesis. It is tempting to draw a parallel between this situation and Holtfreter's[10] theory of the role of sublethal cytolysis in the induction of embryonic neural tissue from prospective epidermis in amphibian embryos—especially since it appears that one of the first tissue types to appear in the development of testicular teratomas of our mice resembles embryonic brain.

Our observations fail to prove but strongly indicate that environmental influences, such as vascular abnormalities, are at least partially responsible for the genesis of testicular teratomas in the genetically susceptible strain 129 mice. The experimental production of testicular teratomas in fowl by injections of salts involves injury to the gonad, and it may be that a specific genetically controlled set of circumstances may also result in injury and consequent teratoma formation in strain 129 mice. This possibility will receive further study.

In humans, testicular teratomas have been found in young males, which indicates a congenital origin. They usually do not become clinically detectable until the fourth decade of life, and it is believed that there is a long period of dormancy, followed by a postpubertal activation of growth. The occurrence of teratomas in very young mice indicates that these tumors, too, may be congenital in origin. As shown in Table 1, the available data indicate that there is no increase in the frequency of these tumors with advancing age. These data suggest that, in these mice, the tumors develop as a result of influences acting at an early (probably prenatal)

developmental stage and that they do not arise in later life. On the other hand, the studies on fowl and the rat demonstrate that the testis is susceptible to postnatal teratogenic influences.[6] The results of the studies with rat testis are not in agreement with those of Li *et al.*,[9] who showed that intrasplenic testicular grafts in castrate mice do not become teratomatous. Our results with intrasplenic testicular grafts, using an unusually susceptible strain of mice, are in accord with the latter findings, which makes it seem unlikely that this particular condition of hormone unbalance promotes the genesis of testicular teratomas in mice.

With one exception, grafts of spontaneous teratomas have persisted but have not as yet developed into progressively growing transplantable tumors. This is in contradistinction to the behavior of grafts of spontaneous ovarian teratomas which have developed into rapidly growing transplantable tumors.[2, 3] As stated above, undifferentiated embryonal cells are infrequently present in teratoma testis of strain 129 mice, which may account for the "benign" behavior of the grafts. The maintenance of progressive growth and pleomorphic character through serial transplant generations of ovarian teratomas has been ascribed to the presence of pluripotent embryonal cells. This view is strengthened by the fact that the single transplantable testicular teratoma exhibiting progressive growth is composed entirely of undifferentiated, rapidly dividing embryonic-type cells.

Teratomas in the testes of very young mice contain undifferentiated embryonic cells, nervous tissue, and cysts lined with cuboidal epithelium. The preponderance of embryonic cells in these relatively simple tumors makes it seem likely that they give rise to the various types of differentiated cells found in the more complex teratomas. This view is in accord with the interpretation of the results of the transplantation experiments of Jackson and Brues[2] and Fekete and Ferrigno,[4] which revealed that the various types of tissues in their transplantable mouse ovarian teratomas stem from embryonic undifferentiated cells rather than from neoplastic differentiated elements. Our observation of "normal-appearing" nervous tissue, bone, cartilage, muscle, fat, etc., along with repeated failure of teratomas composed of these elements to demonstrate progressive growth upon transplantation, does not suggest a multiplicity of tumor types. We seem to be faced with a paradoxical situation in which neoplastic cells differentiate into normal-type tissue cells.

A morphogenetic scheme has been presented by Friedman,[11] in which it is proposed that the following sequence of tumor types prevails in the development of human testicular teratomas: germinoma → "embryonal carcinoma"—*teratogenesis* → teratoma. On the basis of extensive morphological observation, Dixon and Moore[12] deem it most likely that the earliest form of tumor which develops into testicular teratoma is embryonal carcinoma composed of nearly totipotent, undifferentiated cells of presumably germ-cell origin. Our findings are in accord with these theories.

SUMMARY

Testicular teratomas develop in about 1 per cent of strain 129 males. These tumors grow progressively and are composed of many types of embryonic and adult tissues foreign to the testes. Teratomas were observed in very young strain 129 mice, and the incidence does not increase with advancing age. About 5 per cent of 129 strain males exhibit another pathological condition, small testis, characterized

by reduction in size of the testis through degenerative changes. Both teratoma testis and small testis occur about three times more frequently on the left side than on the right, and it is suggested that similar etiological factors, which may be associated with gonadal migration, are involved.

There seems to be a correlation between the size of a teratoma and the number of tissue types it contains. The simplest teratomas examined were composed of undifferentiated embryonic tissue, nervous tissue, and cysts lined with cuboidal epithelium. The more complex tumors contained several additional tissue types. It is suggested that differentiated elements stem from pluripotent embryonic-type cells.

Fifteen spontaneous teratomas have been transplanted. Of these, all have taken, but only one has developed into a rapidly growing transplantable tumor. This transplantable tumor consists of undifferentiated cells which grow both subcutaneously and in the ascitic fluid.

It is pointed out that an inbred strain of mice in which a relatively large percentage of males develop testicular teratomas may be an important tool in the study of some hitherto unexplored aspects of the biology of these interesting growths.

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*PHYSICS AND PHYSIOLOGY OF NEUTRON-CAPTURE THERAPY**

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Neutron-capture therapy is an experimental procedure for achieving selective irradiation of a diseased tissue by inducing radioactivity throughout that tissue.

The radioactivity is induced *in situ* by the capture of thermal neutrons by a suitable target element. For best effects the target element is so chosen that decay of the excited isotope is immediate and results in energetic heavy particles, for example, alpha particles. The advantages of heavy particles are that they have short

ranges in tissue and that they have high relative biological effectiveness. A high thermal-neutron capture cross-section is another essential requirement in selection of the target element. Only three elements decaying by heavy-particle emission have adequately large cross-sections. These are lithium 6, boron 10, and uranium 235, having cross-sections of 950, 3990, and 549 barns.†

The nuclear equations representing these reactions and the energies involved are

$$
\begin{aligned}
{}_3Li^6 \quad + {}_0n^1 &\rightarrow {}_3Li^{7*} \quad \rightarrow {}_1t^3 \quad + {}_2\alpha^4 + 4.64 \text{ mev}, \\
{}_5B^{10} \quad + {}_0n^1 &\rightarrow {}_5B^{11*} \quad \rightarrow {}_3Li^7 + {}_2\alpha^4 + 2.79 \text{ mev}, \\
{}_{92}U^{235} + {}_0n^1 &\rightarrow {}_{92}U^{236*} \rightarrow {}_{\sim 38}Z(97 \text{ mev}) + {}_{\sim 54}Z(65 \text{ mev}).
\end{aligned}
$$

The asterisk in the above equations denotes the excited intermediate state of the compound nucleus formed by neutron capture.

In 1936 Locher[1] suggested the possibility of irradiation by neutron activation *in situ*. The first studies pertaining to the suitability of the boron 10 reaction were reported by Kruger in 1940,[2] and in the same year Zahl, Cooper, and Dunning[3] reported studies involving both boron 10 and lithium 6. In 1948 Tobias, Weymouth, Wasserman, and Stapleton[4] reported studies on biological effects induced in animals by thermal-neutron radiation following administration of uranium, using a reactor as the neutron source.

When the research reactor at Brookhaven National Laboratory began full power operation early in 1951, the clinical program for the treatment of malignancy by neutron-capture therapy was initiated in the medical department. There being no obvious first choice among the three possible capture elements, the availability of highly enriched boron 10 and the extensive medical literature dealing with the toxicology of boron led us to select this element for the first studies of the physiological problems involved in neutron-capture therapy.

From the physiological standpoint the first problem is to achieve a significantly greater concentration of the capture element within the volume to be irradiated than obtains in the surrounding areas, and the absolute concentration must be great enough to permit sufficient irradiation to be achieved in a reasonable time span.

These conditions would be met if a compound were available for which the tumor had a preferential uptake. Such a compound would contain the capture element and would be greatly concentrated from the blood by the tumor. By "preferential uptake" we mean the type of uptake which is seen in the case of iodine when it is concentrated by normal thyroid tissue. However, no such compound is known for tumors today. For this reason, we have developed an alternative approach, which we have termed "selective kinetics." In the application of selective kinetics we capitalize upon the transient differences in concentration resulting from differing rates of transfer of the target element from blood to the several tissues of immediate interest. When this concept is used, we are primarily concerned with concentration differences which exist before equilibrium is reached and are not concerned with final distribution.

The hypothetical relationships between time and changing concentration can be demonstrated in Figure 1, which shows the situation presumed to exist during the experimental application of slow-neutron-capture therapy to glioblastoma multiforme, a malignant tumor affecting the brain.[5] With time plotted from beginning

of the injection, the concentration in plasma rises quickly and then decreases as the borax is taken into various tissues or is removed from circulation by normal eliminative processes. The curve typifying concentration in the brain tumor is seen to follow the rise quickly and then follows the same general decreasing function. In normal brain tissues, however, the concentration of borax comes up quite slowly and reaches equilibrium only after an hour or more. The administration of the radiation treatment is accomplished by application of slow neutrons during this temporary state when a greater amount of the capture element is present in the disease area than in the normal tissues. In the first series of experiments the neutrons were used in the interval from ten to forty minutes after injection.

These differences in concentration arise from differences in circulation and permeability. To establish the validity of these assumptions with regard to a target element it would be most convenient to use a radioactive isotope of that element as a tracer. For boron and lithium no suitable radioactive isotope exists. This difficulty might be obviated by placing the tissues in a neutron beam and determining the instantaneous concentrations of the target element by the use of alpha-particle detectors *in situ*. To date, we have not been successful in developing instrumentation for this purpose. Consequently, we have perforce resorted to the study of other radioactive elements to obtain certain data pertinent to this study and have related these data to chemical determinations of the target element in tissues removed at various time intervals after injection. We have studied boron behavior in transplantable mouse brain tumors induced originally with methylcholanthrene. With reference to selective kinetics, these serially passaged tumors behave apparently in a manner similar to glioblastoma multiforme in man. Representative data from such a study are shown in Figure 2. Equilibrium in the several tissues studied is attained at about seventy-five minutes after injection. The comparison of boron concentration in tumor and normal brain is seen to be well marked. Experimental difficulties due to the smallness of the experimental animal introduce some uncertainty that only undamaged brain is included in the sample, a factor which would serve to decrease the apparent preferential distribution.

In the case of brain tumors, the phenomenon of the blood-brain barrier, by retarding the appearance of boron in normal brain tissue, enhances the selective kinetics, or favorable concentration ratio, since this barrier does not exist in the tumor. If the blood-brain barrier is depended upon to retard distribution through normal brain, it is necessary to determine whether the barrier remains intact following radiation when more than one treatment is contemplated. Although we could not readily study the blood-brain barrier in our patients, we could study the blood–ventricular fluid barrier and have assumed that this would behave in the same manner. Thus far we have not been able to demonstrate any effect upon this barrier by the treatments.

In the first experimental series ten patients were irradiated with thermal neutrons at the Brookhaven reactor. The treatment facility is shown schematically in Figure 3. The upper layer of shielding was removed over an area four feet by eight feet, and a special block was inserted in the lower section. This block is a hollow cone which permits neutrons from a large area of surface of the graphite reflector surrounding the reactor core to be utilized at the two-inch-by-four-inch treatment port. Leakage neutrons escaping from this reflector are thus funneled through the

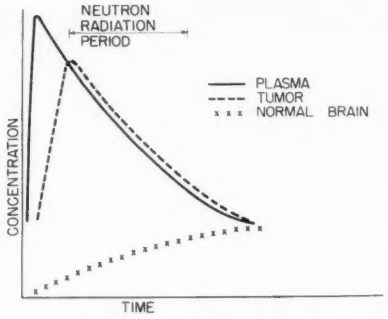

FIG. 1.—Time relationship of boron concentration in plasma, tumor, and normal brain following intravenous injection. The maximum concentration in tumor is shown at approximately twelve minutes postinjection, and a neutron irradiation period of forty minutes is indicated.

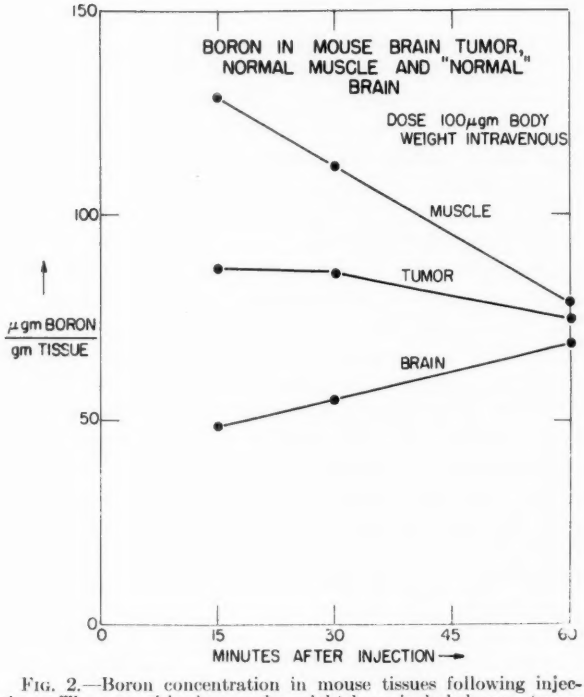

FIG. 2.—Boron concentration in mouse tissues following injection. The normal brain samples might have included some tumor tissue.

volume to be treated. A bismuth shield is interposed to reduce the amount of gamma radiation which accompanies the neutrons.

Studies of the neutron flux were made at this facility, using a phantom. In order to get valid data for the neutron flux, it is necessary that the phantom match tissue not only in its density but also in the atomic population. This was done with special plastic materials and with liquid solutions contained in a thin plastic box. Gold foils were employed as neutron detectors, using the neutron activation technique.[6] The results are shown in Figure 4, superposed on a sketch of the treatment situation. These figures have been substantiated also by intracranial measurements made during treatment with gold wires inserted through the treatment area.[7]

In this study the results obtained were encouraging clinically but were far from definitive. Assessment of the conditions under which multiple radiations were carried out indicated that boron concentration in the tumor area was low and that the attenuation of thermal neutrons was so great as to preclude the possibility of effective treatment in areas approaching the midregion of the head, where effective tumor control is imperative. Two objectives became immediately apparent: (1) to increase the thermal-neutron flux and (2) to increase the boron dose.

During the past year we have redesigned our reactor facility so that now the flux has increased from 2×10^8 to 3×10^9 thermal neutrons per square centimeter per second at the skin surface. Figure 5 shows the new treatment facility. A shutter is included to permit setting up the patient for treatment without requiring the reactor to be shut down. This compound shutter consists of a thin layer of boron carbide to absorb the slow neutrons, several inches of paraffin and lithium fluoride to thermalize and capture the fast neutrons, and two inches of lead as additional gamma-ray shielding. The shutter is also of great value in carrying out the extensive small-animal irradiations which are an important part of the research program. A second major improvement is the increase in neutron flux. This has been achieved by making the treatment level lower, widening the angle of the funnel block, and minimizing the amount of structural material in the path of the neutrons. Also, a treatment port as large as four inches square can now be used. The gamma-ray contamination in the beam has been lessened by removing the aluminum covering normally used on bismuth. We have also carefully evaluated the toxicity of intravenously administered boron in mice and believe we can safely increase the dose over that previously given. During April, 1954, a new series of experiments was begun in which the patients are treated with the higher flux and with larger doses of borax. It is too early to make any statement of the results, but the immediate treatments were successful.

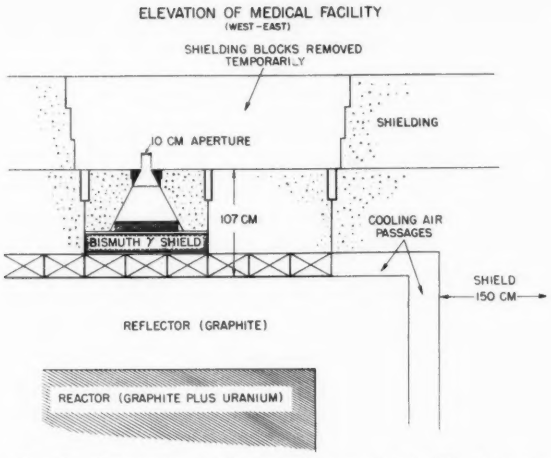

FIG. 3.—Schematic detail of nuclear-reactor facility in cross-section, showing geometric relationship of treatment site, shielding, and radiation source.

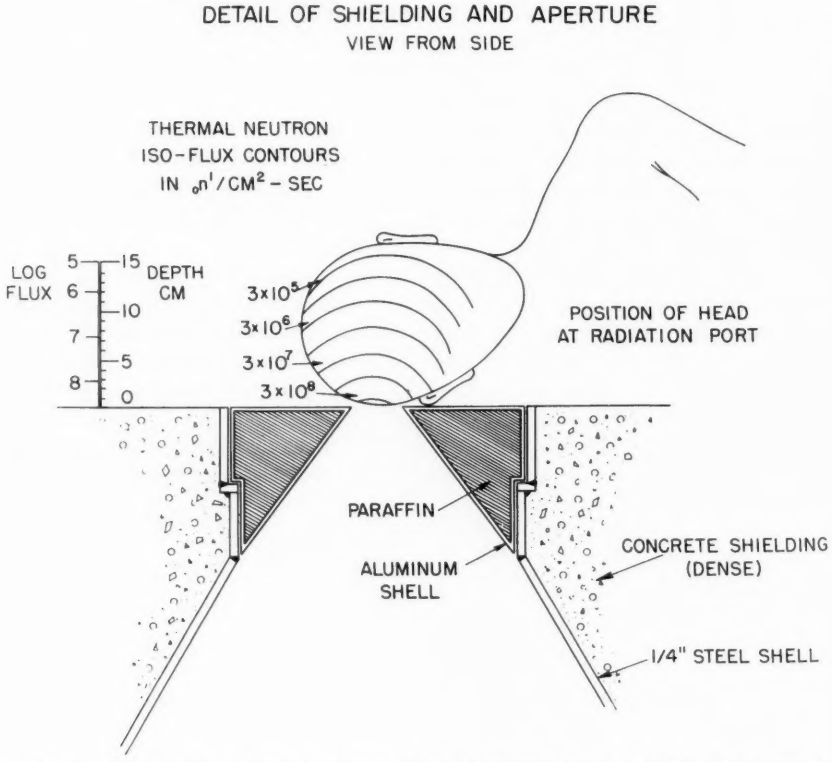

FIG. 4.—Slow-neutron penetration pattern, showing attenuation with depth. This figure is idealized from measurements in phantom and in vivo using gold-foil activation technique.

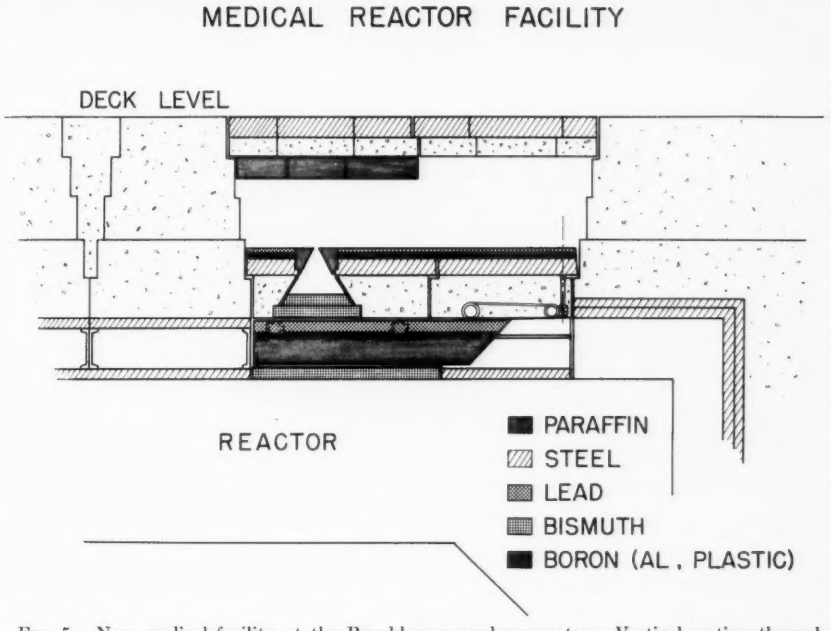

FIG. 5.—New medical facility at the Brookhaven nuclear reactor. Vertical section through center of treatment cone, showing arrangement of shielding materials, compound shutter, and limitation of neutron flux.

Our experience to date points to the desirability of improving the neutron penetration of the deeper-lying tissues, a result which possibly can be achieved by developing a beam providing epithermal as well as thermal neutrons.

Summary.—We have utilized the phenomenon of thermal-neutron capture to develop radiation *in situ* in patients suffering from glioblastoma multiforme. We have applied our concept of selective kinetics to localize this radiation to the sites desired. The results to date have been encouraging and have suggested numerous other basic studies required for fuller development of this therapeutic approach.

* This research was supported by the Atomic Energy Commission.

† The barn is a unit of magnitude 10^{-24} cm². The neutron-capture cross-section expresses the probability of capture in terms of the effective target area which the nucleus presents to the neutron.

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